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# The Elimination of Listeria monocytogenes Biofilms from Stainless Steel Deli Meat Slicer Components by the use of Hurdle Technologies

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# The Elimination of *Listeria monocytogenes* Biofilms from Stainless Steel Deli Meat Slicer Components by the use of Hurdle Technologies

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**Abstract**

*Listeria monocytogenes* growth is of the greatest concern amongst ready-to-eat foods. The United States Department of Agriculture's Food Safety and Inspection Services determined that deli luncheon meats pose the greatest risk of contamination from *L. monocytogenes* Industrial meat slicers have many removable parts that are connected with sealers and gaskets, which can become worn over time. These spaces cannot be cleaned adequately, therefore are susceptible to bacterial growth. Planktonic cells form biofilms in order to protect the cell from adverse conditions, like during routine cleaning and sanitation. Once a biofilm is formed, the bacteria are much more difficult to eradicate and can be more resistant to the lethal effects of chlorine. This study analyzed the biofilm forming abilities of different *L. monocytogenes* serotypes and *L. innocua* by observation through motility tests, microtiter plate biofilm assay and microscopy. *Listeria* strains were grown on stainless steel coupons cut from a deli meat slicer blade in order the observe biofilm growth. This study also investigated the synergistic effects of steam and chemical sanitizers on disrupting and removing the biofilms formed on the stainless steel coupons. Both flagellated and non-flagellated *Listeria* strains produced biofilms and there was no correlation observed between the production of biofilms and hydrophobicity if the films. Overall there was a 5 to 7 log reduction between the combined treatments and the initial inoculation. The sanitizer alone gave a 2 to 3 log reduction and the steam treatment resulted in a 3 to 4 log reduction. The results of this study will provide better understanding of and potential methods for the sanitization of deli meat slicers. In turn, the knowledge gained from this study will reduce the risk of contamination and outbreaks of *L. monocytogenes* and other food-borne pathogens.

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**Dedication**

I dedicate this work and give special thanks to my best friend and love, Brandon. You have been supportive of me and patient with me throughout the whole process. Special thanks also go to my parents, Deborah and Michael Mertz, who did all they could to get me here today. They always pushed me to reach my potential, and for that I will always be thankful. To my sister and friend, Michaela Mertz, thank you for bringing me joy and laughter throughout my studies. This project would not have been possible without all of the loving support and encouragement I received from my family and friends.

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# Chapter I

## General Introduction

### Statement of Problem

In recent decades *Listeria monocytogenes* has been recognized as an emerging food-borne pathogen. Upon eating a food contaminated with *L. monocytogenes*, the bacterium disseminates in the intestines and can enter the central nervous system, resulting in listeric infection. The symptoms of listeriosis vary greatly from those commonly associated with food poisoning, manifesting itself as meningitis, septicemia, fever and eventually death (Rocourt, 1996; Bell and Kyriakides, 2009). An estimated $152 billion a year cost on the United States health care results from expenditures on both acute disease and long term care of patients who contract listeriosis (Scharff, 2010).

*L. monocytogenes* growth is a concern amongst ready-to-eat (RTE) foods, which are not heated before consumption. In a study conducted by the United States Department of Agriculture (USDA) Center for Food Safety and Applied Nutrition and the USDA's Food safety and Inspection Services (FSIS), it was determined that deli luncheon meats pose the greatest risk of contamination from *L. monocytogenes*. The Center for Disease Control and Prevention (CDC) estimate there are 1600 cases of listeriosis a year, 260 that result in death (CDC (a), 2014). Deli meats sliced at retail have proven to have as much as 7 fold higher prevalence of *L. monocytogenes* contamination than meats sliced within a USDA inspected facility (Gombas *et al.*, 2003). The report by Gombas *et al.*, (2003) further concluded that current cleaning and

disinfecting practices for deli slicers are inadequate, therefore posing high risks for cross contamination.

**Study Justification**

In order to observe *L. monocytogenes* biofilm development on deli meat slicers, stainless steel coupons (cut from a deli meat slicer blade) were inoculated with each strain. According to United States Food and Drug Administration (US FDA) regulation, slicers used in retail should be cleaned and sanitized at least every 4 hours when used at room temperature (Chavant *et al*., 2004). To replicate a situation similar to the industry, the inocula had a contact time of 4 hours. Sanitizers and disinfectants cannot work effectively to penetrate the biofilm matrix if the surface still has particulate matter left after an ineffective cleaning process (Simões *et al*., 2010). Effective cleaning processes should disrupt the extracellular polysaccharides (EPS) matrix of the biofilm so the sanitizers can have access to the cells within (Simões *et al*., 2006). The ideal sanitizer should be effective, safe, easy to use, not corrode the surface being disinfected and not leave any toxic residues. Heat has also proven to be an effective form of sanitization (Trivedi *et al*., 2008). In a study by Crandall *et al*., (2012) heating the components of the deli meat slicer, inoculated with *L. innocua,* under moist heating conditions caused a 5 log reduction within 3 hours at 82˚C. In the same study, the sanitizers used only delivered 1.0 to 2.0 log CFU/ coupon reduction.

Steam allows for a large amount of heat to be transferred. During the condensation of steam on a food contact surface, the surface is heated very rapidly (James *et al.,* 2000). At 100˚C, steam has a greater heat capacity than water (James and James, 1997). Steam has the capability to penetrate cracks and crevices that standard cleaning methods cannot (Morgan *et al.,*

1996). It would be expected that when two methods of sanitation are applied, the lethality on microorganisms would be greater than if one is applied alone; this combination of treatments is referred to as hurdle technology (Leistner, 2000). In a study by Ban *et al.* (2012), steam was used in conjunction with lactic acid. The use of the two treatments together proved to be more potent in killing *L. monocytogenes* than when each treatment was applied separately. Through this current study, it is determined if steam, sanitizers, or a combination of the two prove to be the most effective treatment to eradicate *L. monocytogenes* from stainless steel processing equipment.

**Study Objectives**

The first objective of this experiment was to analyze and understand the biofilm formation abilities of *L. monocytogenes* and *L. innocua*. To understand the biofilm formation, each strain was compared to known biofilm formers *Pseudomonas aeruginosa* and *P. fluorescens*. It was hypothesized that the flagella, present on the surface of *Listeria*, assist with the development of biofilms. Therefore, each strain was first observed for motility. The biofilm forming abilities of each cell were quantified via microtiter biofilm assay. The cellular surface hydrophobicity was also observed to determine if there was any correlation between hydrophobicity and the amount of biofilm development.

The second objective of this experiment was to analyze the effects of combining two sanitation methods in order to effectively remove bacterial growth and biofilm development. In this experiment, steam and a commonly used industrial sanitizer were analyzed for their ability to disrupt the biofilm matrix formed by *L. monocytogenes* and *L. innocua*. To understand if steam and sanitizer together are the most effective method, stainless steel coupons inoculated

with *Listeria* strains were tested after the bacterial contact time, the rinse step, use of sanitizer alone, use of steam alone and use of steam with the sanitizer. Thermocouples were used during the steam treatment in order to determine time, temperatures and relative humidity. With the data collected, the percent cells recovered was determined by standardizing the colony forming units per centimeter squared (CFU/ $cm^2$) recovered after each treatment with the CFU/ $cm^2$ recovered from coupons only treated with deionized water.

**Context of Study**

This study is a follow-up to a project analyzing cost effective treatments that reduce the risks of *L. monocytogenes* contamination of ready-to-eat deli meats prepared in retail delis. The first study, within the project, focused on a visual verification system that helped to ensure the food contact surfaces were clean. The study aimed at improving sanitation methods to ensure *L. monocytogenes*, both planktonic cells and enclosed cells, were effectively removed. Within this project, the effectiveness of sanitizers used typically in retail settings were assessed. Studies were also conducted utilizing the effectiveness of bread proofing ovens as a sanitation method for deli meat slicers. These studies were used to determine the most effective temperature and time needed to achieve a significant log reduction. The follow-up was conducted to combine and utilize the data collected throughout the project; specifically analyzing the effectiveness of applying both sanitizers and steam.

**Research Question and Hypothesis**

Cross-contamination is a serious concern in ready-to-eat retail deli meat. Conventional cleaning and sanitizing methods are not effective for removing *Listeria* biofilms. This study was aimed at determining a more effective sanitation method for industrial slicers, in the hope of

reducing cross-contamination. In order to improve upon the current sanitation methods, more research needed to be conducted on *L. monocytogenes* biofilm forming characteristics. This study is focused on 3 main questions:

1. Does biofilm development depend on the presence of flagella?
2. Is there correlation between biofilm development and cellular surface hydrophobicity?
3. Is it possible to achieve a 5 log reduction of a cocktail of *Listeria* strains, inoculated on coupons cut from deli slicer components, by subjecting them to sanitizers at 5 to 25 ppm and to moist heat at 40˚C and 47˚C?

We hypothesize that:

1. *Listeria* cells having flagella will be able to attach more readily to the coupons than those without flagella. Biofilm development begins with attachment; therefore cells that attach more readily will have more opportunity to develop biofilms.
2. In previous studies, cellular surface hydrophobicity was found to dictate the attachment and biofilm development capabilities of *Listeria* strains on PVC and fruit surfaces. Cellular surface hydrophobicity is a factor in biofilm development on stainless steel and aluminum slicer components.
3. The sanitizers and steam treatments were applied at lower concentrations and temperatures (respectively) than those found to be effective in previous studies. The two treatments could be decreased because they were used in combination with each other, and therefore it is believed that a 5 log reduction of a cocktail of the *Listeria* strains will be achieved.

**Assumptions**

This study was based on the following assumptions:

1. If a cell proved to be motile, then flagella are present.
2. The shelf of the bread proofing oven represents the outer surfaces of the deli slicer. This area will have direct access to steam and should reach the oven proofer temperatures before the internal compartments of the deli slicer.
3. The motor compartment (MC) of the deli slicer represents the "cold spot" in the deli slicer and that it is the last to reach the oven proofer temperature and have indirect access to steam. This area represents niches of the slicer that may be more difficult for the food service staff to clean efficiently.

**Limitations**

1. The microtiter biofilm assay estimates the biofilm development on PVC and not stainless steel or aluminum.
2. The biofilm assay quantified biofilm development after 24hr and 48hr. A deli meat slicer is disassembled and cleaned every 4hr of use. Biofilm development after 4hr is not known or can be concluded from this study.
3. The concentration of the sanitizers was determined based off of the use instructions and concentration test strips specialized for each sanitizer.
4. The *Listeria* strains were grown in tryptic soy broth with 0.6% yeast extract (TSBYE) and re-suspended in phosphate buffered saline (PBS) rather than a food matrix. Using a food matrix would have been more representative of a real life situation where lipids,

carbohydrates and proteins are present and facilitate in bacterial proliferation and biofilm development.

5. The motor compartment is used as a representative for all the hard to reach areas of the slicer. However, it can only be used as an simulate. Seals, worn gaskets and seams can accumulate food debris and bacteria, creating a niche for *L. monocytogenes*. These conditions are not possible to create in controlled settings.

**Organization of This Study**

This thesis contains four chapters. Chapter 1 is an introduction to the purposes and justifications of the study, contexts, assumptions, limitations, objectives and hypotheses. Chapter 2 includes the review of literature and studies that analyzed listeriosis, growth in extreme conditions, biofilm development, sources of contamination and the cleaning and sanitizing of food contact surfaces. Chapters 3 and 4 are descriptions of studies analyzing the biofilm forming characteristics of various *Listeria* strains and the elimination of *L. monocytogenes* from deli meat slicer components by combining steam and sanitizers.

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Chapter II

# Review of Literature

## General Characteristics of *Listeria monocytogenes*

*Listeria monocytogenes* is a ubiquitous bacterium of the family Corynebacteriaceae, order Eubacteriales (Pirie, 1940; Gray and Killinger, 1966). *L. monocytogenes* is a Gram positive, non-spore forming, facultative anaerobic, intracellular pathogen with a diptheroid-like rod structure and approximately 1.0-2.0 µ by 0.5 µ in size (Smith and Metzger, 1962; Gray and Killinger, 1966; Walker and Stringer, 1987; Junttila *et al*., 1988; Farber and Peterkin, 1991; Portnoy *et al.* 1988). *Listeria* is catalase positive, oxidase negative and expresses β-hemolysis (Christie *et al.*, 1944; Farber and Peterkin, 1991). β-hemolysis produces zones of clearing on blood agar. Hemolysin production is essential for the growth of *L. monocytogenes*, and therefore is essential in the differentiation of *Listeria* spp. (Portnoy *et al.* 1988; Dominguez Rodriguez *et al*., 1986).

*L. monocytogenes* have peritichous flagella, which allow for a tumbling motility. The flagella are tightly coiled or spiral-like. The average length is 2.01 µ with amplitude of 0.48 µ. Each cell has anywhere from 4 to 6 peritichous flagella and each flagellum has thousands of flagellin monomers (Vatanyoopaisarn *et al.*, 2000). Flagella play a role in biofilm formation. Flagella are the transport system of the cell that allow for the initial cell to surface interactions necessary for attachment to the surface (Harbron and Kent, 1988).

However, motility may not always be evident when analyzing *L. monocytogenes* cells. The flagella are only present under a narrow temperature range (20 to 30˚C). Below 30 ˚C, the

*mogR* gene is inhibited by an antirepressor GmaR, allowing for flagellar gene transcription. Inversely, above 37 ˚C, the *mogR* gene represses the transcription of the flagellar gene making the *L. monocytogenes* cells non-motile. This means *L. monocytogenes* will develop flagella at room temperatures but not at mammalian body temperatures (Peel *et al*., 1988). To test for the presence of the flagella, a semisolid motility test is performed. In a positive test, an "inverted pine tree effect" is observed (Peel *et al.*, 1988; Farber and Peterkin, 1991).

*L. monocytogenes* grows well in tryptose agar/ tyrptic soy broth supplemented with 0.6% yeast extract and incubated at 30˚C. When *L. monocytogenes* colonies are viewed with a binocular scanning microscope, with the use of obliquely transmitted light, two observations can be made: (i) the colonies have a textured surface and (ii) the colonies exhibit a blue-green sheen. In tryptic soy broth, *L. monocytogenes* produces clouding within 18 to 24 hr. After several days, a thick, sticky slime precipitate forms in the liquid medium. Growth can be increased by the addition of a fermentable sugar, such as glucose. Growth is optimized under anaerobic conditions (Evans *et al.*, 1985; Farber and Peterkin, 1991).

**Discovery of *L. monocytogenes***

The basic description of *L. monocytogenes* was not recorded until 1911 when a Swedish worker, Hulphers, isolated it from necrotic foci in the liver of a rabbit. Hulphers named the organism *Bacillus hepatis* (mostly likely because of the specimen's rod shape and its isolation in the liver). His description accurately reflects what is now known to be *L. monocytogenes* (Gray and Killinger, 1966). In 1926, Murray *et al.* (1926), isolated the bacterium from the liver of sick rabbits and guinea pigs and named it *Bacterium monocytogenes.* Within the next year, Pririe (1940), isolated an identical bacterium from the liver of gerbils. However Gill, in New Zealand,

was credited with the first isolation of *L. monocytogenes* in domesticated farm animals, referring to it as *Listeric encephalitis*. The first case of listeriosis recorded in man was made by Nyfeldt in 1929 when the bacterium was isolated from three patients in the United States (Gray and Killinger, 1966). There was considerable confusion about this bacterium's name until the Third International Congress for Microbiology convened in 1939, where the name *Listeria* (in honor of Lord Lister, a pioneer in microbiology) *monocytogenes* (originally suggested by Murray *et al.*, derived from the fact that monocytes are often found in the peripheral blood) was designated. The name *Listeria monocytogenes* was first used in 1940 in the Sixth edition of Bergey's Manual of Determinative Bacteriology (Gray and Killinger, 1966). *L. monocytogenes* has been recognized as an emerging food-borne pathogen since the early 1980s (Samelis and Metaxopoulos, 1998).

### *Listeria* Infection

*L. monocytogenes* is not only a concern because of its ability to thrive in extreme conditions, but because of the infection it causes. Listeriosis is defined as "a patient with a compatible illness from whom *L. monocytogenes* was isolated from normally sterile blood or cerebrospinal fluid" (Gillespie et al., 2006; Bell and Kyriakides 2009). After *L. monocytogenes* has been consumed, the bacteria systematically disseminates from through the lumen in the intestines to the central nervous system. The bacteria are able to cross the intestinal barrier because of the cellular surface protein- internalin (InlA) (Lecuit *et al.*, 2001). Listeriosis accounts for an estimated 1600 illnesses and 260 deaths a year, with a mortality rate of 25% (CDC, 2013; US FDA, 2002).

Typically, healthy individuals will not contract listeriosis upon consumption of contaminated food, however gastroenteritis will most likely occur. People most at risk for contracting a listeric infection are organ transplant patients, patients with HIV/AIDS, patients with immune-compromising diseases, pregnant women, patients with cancer, children, and the elderly. The symptoms associated with listeriosis differ from those typically associated with food poisoning and vary widely depending on the patient's age and the onset time of the infection (Rocourt, 1996; Bell and Kyriakides 2009).

The bacteria will typically infect the uterus of pregnant women, the bloodstream, or the central nervous system. In pregnant women, the infection may result in stillbirth, spontaneous abortion, or the birth of an extremely ill baby. The mother herself is very rarely affected by the disease. The infection primarily attacks the fetus. In newborns, the infection can be acquired postnatal from either the mother. There are two forms of neonatal listeric infection: early-onset and late-onset. Early-onset occurs while the fetus is still in the uterus. The primary disease associated is septicemia; however, respiratory distress, cyanosis, apnea, pneumonia and microabscesses are also seen. The mortality rate is 15 to 50%, and the child is usually born premature with a low birth weight. Late-onset is when the infection occurs after birth. The infant becomes infected from the mother at birth or from cross-infection with another neonate. The primary disease associated is meningitis; however, fever, poor feeding, irritability, leukocytosis and diarrhea have also been observed. Most neonatal deaths from listeriosis are due to respiratory failure and pneumonia (McLauchlin, 1990; Farber and Peterkin, 1991).

In non-pregnant adults, the immunocompromised, and the elderly are the most at risk because of the decreased ability of their immune systems to fight off infections. Listeriosis will most likely manifest as meningitis, or septicemia (Rocourt, 1996; Bell and Kyriakides, 2009).

However, listeriosis can also occur as: meningoencephalitis, endocarditis, endophthalmitis, osteomyelitis, brain abscesses, peritonitis, diarrhea, fever and death (Gray and Killinger, 1966).

The type and severity of the illness is partially related to the onset time of the infection and the age and condition of the patient. Listeric infection has an incubation time of 1 to 2 days. Its primary symptoms are self-resolving skin lesions. However, if left untreated it may result in meningitis and eventually death. Listeriosis is an infection that occurs in non-pregnant adults and its incubation time varies from 1 day to several months. The patient may be asymptomatic or present with mild illness which will progress to more severe illnesses or central nervous system infections (meningitis or septicemia). *Listeria* induced food poisoning is caused by the consumption of foods containing extremely high levels of *L. monocytogenes* (greater than $10^7$ /g). Its incubation time is relatively short (less than 24 hr). The primary symptoms include vomiting, diarrhea and fever. Food poisoning caused by *Listeria* is typically self-resolving (Rocourt, 1996).

Listeriosis and listeric infections take a toll on US healthcare, estimated at $152 billion per year. That accounts for the costs of acute diseases and long-term care for patients who contract listeriosis. *L. monocytogenes* has the highest costs of long-term care compared to illnesses due to other food-borne pathogens (Scharff, 2010). *L. monocytogenes* ranks second only to *Vibrio* in costs to treat a single case. *L. monocytogenes* is the third highest in costs for treatments of a single food-borne pathogen; with *Campylobacter* and *Salmonella* ranking above it (Scharff, 2010). Between 2006 and 2008, there were on average 3 cases/ million persons of laboratory confirmed *Listeria* infections reported (CDC (b), 2014). The 2010 Healthy People goal was to decrease that number to 2.4 cases/ million persons (CDC, 2008). The 2020 Healthy People goal is to decrease the number to 2.0 cases/ million persons (CDC (b), 2014). However,

the most recently available statistics show that the level of listeria infections remains at 2.9 cases/million persons (CDC, 2013).

### *Listeria* species

There are six different species of *Listeria*, however only two are of particular concern in the food industry, *L. monocytogenes (*pathogenic) and *L. innocua* (non-pathogenic). These two strains do have similar and different characteristics (Boerlin *et al.*, 1992; Bell and Kyriakides 2009). As previously stated, *L. monocytogenes* expresses β-hemolysis; this bacterium also can ferment L-rhamnose but not D-xylose or D-mannitol. *L. innocua*, on the other hand, does not express β-hemolysis. Similarly to *L. monocytogenes*, *L. innocua* does not ferment D-xylose or D-mannitol. The results are inconclusive as to whether *L. innocua* can ferment L-rhamnose (Jay, 1997; Bell and Kyriakides 2009). Therefore *L. innocua* can be used as a model organism for *L. monocytogenes* (Omary *et al*, 1993).

Both *L. innocua* and *L. monocytogenes* have the ability to survive extreme conditions, such as high salt concentrations, extreme pH and temperature changes (Lecuit et al., 2001). *L. monocytogenes* can grow in salt concentrations as high as 12 to 13% with water activities as low as 0.9 (Samelis and Metaxopoulos, 1998). In a study by Cole *et al.* (1990) *L. monocytogenes* was able to grow in higher salt concentrations than most food-borne pathogens. It was also observed that as the temperature increased, so did the ability of *L. monocytogenes* to tolerate higher salt concentrations. At 5˚C, growth was seen in salt concentrations as high as 8%. At 10˚C, growth was seen in concentrations as high as 10%. When the temperature was increased to 30˚C, growth was observed at concentrations as high as 12%. *L. monocytogenes* responds to

the stress of high salt concentrations by producing elongated cells (Hazeleger *et al.*, 2006; Bell and Kyriakides 2009).

**Growth in Extreme Conditions**

*L. monocytogenes* is able to survive extreme pH levels that would kill most other food-borne pathogens. It grows well in a pH range of 4.5 to 7.0. No growth is observed at or below 4.0 (Buchanan and Phillips, 1990; Farber and Peterkin, 1991). However, *L. monocytogenes* has been observed to grow in a pH as high as 9.6 and die at a pH of 5.6. Acetic acid (when used to lower the pH) was the most effective, when compared to the other treatments tested, at inhibiting growth (Gray and Killinger, 1966). *L. monocytogenes* can grow between -0.4˚C and 50˚C with an optimum temperature range between 30 to 37˚C. At 37˚C, growth peaks at 16 to 18 hours of incubation (Gray and Killinger, 1966). *L. monocytogenes* is more heat-resistant than other non-spore forming food-borne pathogens. Its increased tolerance is partially attributed to the rising generation of heat shock proteins and the modifications of the fatty acid profile of the cellular membranes. These are evolutionary modifications made by the bacteria in order to respond to the heat stress conditions (Samelis and Metaxopoulos, 1998). *L. monocytogenes* has cold stress responses that allow the organism to continue to proliferate at low temperatures. These responses include: (i) changes in the cell membrane structure that maintain lipid fluidity and structural integrity, (ii) cells accumulate cryoprotective osmolytes and peptides to maintain enzyme activity, (iii) alterations occur to the cell's surface proteins that allow access to the environment which offers a greater potential for survival for nutritional reasons, (iv) cells produce "cold shock" proteins that protect against oxidative stress and (v) structural changes occur that maintain the functional and structural stability of ribosomes (which are crucial for

protein synthesis) (Bell and Kyriakides 2009). The rising concern of *L. monocytogenes* as a contaminant in food products is its ability to survive such extreme conditions.

**Mechanisms and Function of Biofilms**

Biofilms are composed of an assembly of microbial cells that are irreversibly linked with an enclosed polysaccharide matrix. The matrix may also contain materials such as lipids and proteins collected from the surface where the biofilm forms. The primary function is to protect the bacterial cell from adverse environments and conditions (Breyers and Ratner, 2004). Cells within the biofilm differ from cells in their planktonic form by the genes that are transcribed. Microorganisms can form biofilms on a variety of surfaces, including natural aquatic systems, drains and drain pipes, living tissues, and food contact surfaces (Kumar *et al.*, 1998). Planktonic cells attach in the interface between the surface and the bulk aqueous medium. The processes of biofilm formation are not fully understood (Donlan, 2002).

**Bacterial Cell Attachment**

The attachment of cells to food contact surfaces depends on the adhesion surface, the bulk fluid that transports the planktonic cells and the cellular properties. Cells attach more readily to rough textured, hydrophobic surfaces. The roughness of the surface decreases the shear forces and increases the available surface area (Donlan, 2002). Interactions occur between non-polar, hydrophobic (Teflon and plastics) surfaces, the substratum and the cells that allow the cells to overcome repulsion forces (Kumar *et al.*, 1998; Sutherland, 2001). Food contact surfaces are in constant exposure to liquid media which contain water, carbohydrates, fats, proteins and other nutrients. The aqueous mixture conditions the surface and coats it with polymers which can affect the rate of cellular attachment. A hydrodynamic boundary layer occurs between the

substratum and the liquid medium. The thickness of the boundary depends on the linear velocity. As the velocity increases, the boundary will decrease and cells will experience greater turbulence. Higher velocities result in more rapid contact with the food surface and therefore more rapid attachment. However, if velocities are too high, it will result in the detachment of cells from the surface (Donlan, 2002; Simões *et al.*, 2010).

Based on the cells motility, the attachment of planktonic cells may occur passively or actively. Passive attachment occurs by diffusion, fluid movement and gravity while active attachment is driven by the cell surface (Kumar *et al.*, 1998). Active attachment is typically facilitated by flagella on the surface of the bacterial cell. *L. monocytogenes* can adhere both passively and actively. When *L. monocytogenes* cells are grown between 20-30°C, flagella are present and give the surface of the cell a negative charge (Briandet *et al.*, 1999). As previously stated, the flagella allows for the cell to have motility, which allows for initial interactions between the cell surface and the substratum. However, increased attachment has been observed at a microorganism's highest metabolic activity. Therefore the optimum conditions for *L. monocytogenes* attachment are at 30°C and pH 7 (Herald and Zottola, 1988; Hood and Zottola, 1997). The increased attachment at higher temperatures is due to the heat-shock proteins produced on the surface of the cell when under stress (Samelis and Metaxopoulos, 1998) which suggests that attachment is controlled by surface proteins rather than the presence of flagella, indicating that the function of the flagella is to simply bring the cell to the surface for attachment (Briandet *et al.*, 1999).

Initial attachment of the cell to the substratum occurs within 5 to 30 seconds (Mittelman, 1998). At first, the attachment is reversible because the interactions and forces between the substratum and the bacterial cell are weak. The interactions between the two surfaces involve

van der Waals and electrostatic forces as well as hydrophobic interactions. During this stage of attachment, the bacterial cells still maintain Brownian motion and therefore can be easily removed with mild shear force (Sutherland, 2001).

**Biofilm Formation**

It is known that biofilm formation occurs in a series of steps which allow the microbial cell to come in closer contact with the surface and attach to it firmly allowing for cell-cell interactions. The interactions create a complex structure that is difficult for sanitizers to penetrate. Biofilm formation proceeds as follows: (i) Microorganisms are first pre-conditioned by other macromolecules present in the bulk liquid or on the surface. It has been observed that attachment dramatically increases on surfaces that have been preconditioned with the presence of ions (Barnes *et al.,* 1999; Briandet *et al,*. 1999; Stanley, 1983). (ii) Planktonic cells are then deposited from the bulk liquid to the surface. (iii) Next cells are adsorbed at the food surface contact surface. (iv) The adsorbed cells desorb from the surface. (v) An irreversible link occurs between the cells. (vi)Cell to cell interactions can then occur by the production of signaling molecules. (vii)Substrates are transported to and within the cell, allowing for replication, growth and extracellular polymeric substrates (EPS) formation (Breyers and Ratner, 2004). EPS allows for cells to bind with other particulate material and the surface (Allison, 2003; Simões *et al.*, 2010). Polysaccharides and proteins make up 75 to 90% of EPS composition (Tsuneda *et al.*, 2003). In lesser amounts, nucleic acids and phospholipids substances comprise bacterial EPS structure (Jahn and Nielson, 1998; Sutherland, 2001; Simões *et al*., 2010). (viii) Polysaccharides are secreted by the cells forming a complex matrix. (ix) Biofilms are removed by sloughing or detachment. Once biofilms have broken from the substratum, the vegetative cells within the EPS can recontaminate the substratum (Figure 1) (Breyers and Ratner, 2004).

**Sources of Contamination**

*L. monocytogenes* is predominantly found to reside on complex machinery with small spaces and narrow openings. *L. monocytogenes* has frequently been isolated from slicing, dicing, packaging and brining machinery (Lundén *et al.*, 2002). In a study by Autio *et al.* (1999) the areas of highest contamination in a cold-smoked rainbow trout facility were in areas dedicated to brining, slicing and packaging. *L. monocytogenes* was not detected in either the arrival or departure areas. However, *L. monocytogenes* was detected in the drains of the slicing and packaging areas before and during processing. The gloves of employees working on the production line after brining tested positive for *L. monocytogenes*, while those pre-brining tested negative. These researchers concluded that the two major sites of contamination were related to brining and slicing.

The complex machinery in a food processing plant is difficult to clean efficiently, therefore allowing *L. monocytogenes* to adhere and form a biofilm. The bacteria's adherence increases its ability to resist mechanical and chemical stressors (Lundén *et al.*, 2000). The relocation of processing machinery from one plant to another may also contribute to *L. monocytogenes* contamination (Lundén *et al.*, 2002). The overall design of a processing line may contribute to the repeated contamination of food products. Compartmentalizing the line, by ensuring complete separation of the raw from the post-heat treatment area, is required to limit any cross-contamination. If compartmentalization is poor, then contamination will be persistent (Lundén *et al.*, 2003). Lubricants used in the food industry may also lead to the spread and proliferation of *L. monocytogenes*. In a study by Aarnisalo *et al.* (2003) it was demonstrated that *L. monocytogenes*, although reduced over time, can survive in synthetic lubricants- particularly

those used for conveyor belts. *L. monocytogenes* was transferred from the stainless steel food contact surfaces and into the lubricants.

*L. monocytogenes* adheres to stainless steel, buna-n-rubber, plastics, resins and polypropylene. These materials have uneven surfaces, organic residues, neutral pH and easily absorb water from the surroundings (Lundén *et al.*, 2002; Chasseignaux *et al.*, 2002). Persistent strains of *L. monocytogenes* have been shown to more effectively adhere to stainless steel surfaces after a short contact time than non-persistent strains. Persistent strains are also more resistant to benzalkornium chloride, increasing the bacteria's ability to survive (Lundén *et al.*, 2002). It has been observed that certain strains of *L. monocytogenes* persist and thrive in food processing areas while other strains do not. Lundén *et al.* (2002) recovered 596 *L. monocytogenes* isolates from food processing over several years as a part of a quality control program. All plants observed had persistant and non-persistant *L. monocytogenes* strains. Isolates were identified by their pulse-field gel electrophoresis patterns. Overall, non-persistent strains were isolated from single points in a processing line. The persistent strains, however, were isolated at multiple points on a processing line. This supports the theory that persistent *L. monocytogenes* strains possess qualities that promote growth in a food processing setting, while the non-persistent strains do not. Serotype 1/2c was observed to adhere in food processing environments in the highest numbers. This strain has a different flagellar antigen than the other serotypes observed. In *Pseudomonas aeruginosa* and *Escherichia coli* the flagella was shown to have an effect in the initiation of adherence. The non-motile strain of serotype 1/2c expressed the lowest amount of adherence at short contact times according to the study (Lundén *et al.*, 2000).

Studies have shown that biofilms of *L. monocytogenes* are more resistant to the lethal effects of chlorine than cells in suspension (Lundén *et al.,* 2002; Bell and Kyriakides, 2009). Any solid surface in contact with water and nutrients are subject to microbial colonization, making food processing environments ideal. Biofilm formation on stainless steel has been shown to occur within as little as 2 to 4 hr with virulent strains of *L.* monocytogenes. Microorganisms that form biofilms are as much as 1000 times more resistant to toxic substances and sanitizers than planktonic cells. The high resistance of adhered cells is due to the slower diffusion of the sanitizers and antimicrobial agents through the biofilm matrix, making it more difficult to reach the deeper layers of the biofilm (Krolasik *et al.,* 2010).

The increased use of poultry meat has also contributed to the elevated levels of *Listeria* in processing plants. Persistent *L. monocytogenes* strains collected from poultry processing plants were observed to adhere at short contact times in higher numbers than the persistent strains collected from ice cream processing plants, although the persistent strains at both facilities adhered in higher numbers than the strains considered to be non-persistent (Lundén *et al.*, 2000). In various studies, approximately 16% of raw pork samples and 17% of raw poultry samples were shown to be contaminated with *L. monocytogenes* (Jay, 1997). In a study by Chasseignaux *et al.* (2002), 497 samples were examined (263 which were during processing and 234 after cleaning operations) at two different poultry processing facilities and three different pork processing facilities. Almost 24% of all samples were contaminated by *L. monocytogenes*. During processing, 38% of samples tested positive for *L. monocytogenes*. The percent contamination was almost equal between the pork (37%) and poultry (38.9%) facilities. After cleaning, the percent contamination decreased drastically to 7.7%, with 13.1% contamination on samples from poultry facilities and 2.5% contamination on samples from pork facilities. This

evidence further suggests higher incidences of persistent *L. monocytogenes* strains in poultry and the corresponding processing facilities.

Many human listeriosis outbreaks have been reported from the contamination of foodstuffs such as dairy, processed meats, and other RTE foods. *L. monocytogenes* can survive in dry sausage and grow well in cooked meats and highly acidic poultry products. In a quality control study in Greece, it was observed that 13.3% of vacuumed-packed cooked sliced ham and 20% of cured pork shoulder (samples randomly selected) were contaminated by *L. monocytogenes* (Anonymous, 1995; Samelis and Metaxopoulos, 1998). A study by Samelis and Metaxopoulos (1998) in Greece, on the incidence of *Listeria* species and *L. monocytogenes* contamination in processed meats, discovered that 23.3% of sliced vacuumed-packed cooked meats and 40% of country style sausages analyzed were contaminated with *L. spp*. Also in 6.7% of vacuumed-packed cooked meats and 10% of country style sausages tested, *L. monocytogenes* was present. In this same study, no *L.* species were detected in sausages heated to their final packs or in the fully ripened salamis. This indicates that contamination occurred in handling post-heat treatment in the cutting room. Food-processing equipment, dicers and slicers in particular, which manipulate cooked meats, are most frequently associated with attached *L. monocytogenes*. Once adhered, it is very difficult to eradicate because adaptive responses have occurred. This allows for recontamination on the processing line (Lundén *et al.*, 2002).

In recent decades the presence of *L. monocytogenes* in RTE foods that are not heated before consumption has been a growing concern. Current trends in the food industry are to manufacture convenient RTE foods lower in sodium and other preservatives (Aarnisalo *et al.*, 2003). These factors all increase the likelihood of bacterial growth and potential for causing food-borne illnesses such as listeriosis. The three largest listeriosis outbreaks in the US were

linked to: (i) soft cheese made with unpasteurized milk in 1984, (ii) hotdogs produced in processing plants in 1998 and (iii) sliced turkey meat produced from 2002 to 2003. In a study by Meldrum *et al.* (2010) *L. monocytogenes* was detected in 27 of 950 (2.84%) sandwiches tested from hospital cafeterias in Wales. One sandwich contained extremely high levels of *L. monocytogenes* (1200 colony forming units/ gram (cfu/g)).

Currently the US FDA has established a "zero-tolerance" for the presence of *L. monocytogenes* in a 25 g sample for RTE foods (Czuprynski, 2005). The USDA Center for Food Safety and Applied Nutrition and the USDA's Food Safety and Inspection Services (FSIS), in 2000, conducted an assessment of *L. monocytogenes* contamination in 23 common RTE foods and its risk to public health. Among the RTE foods assessed, deli luncheon meats were found to pose the greatest risk of contamination (FSIS/USDA, 2003).

Approximately 83% of listeriosis cases contracted from contamination of luncheon meats can be attributed to deli meats sliced at the retail deli stores (Kause, 2009). In a study by Garrido *et al.* (2009), *L. monocytogenes* was reported to be in 8.5% of samples from meats sliced and packaged by the retail store, while only 2.7% of samples from meats commercially packaged tested positive, indicating the prevalence of *L. monocytogenes* in store sliced deli meats is 3 fold greater than those pre-packaged by the manufacturer. The USDA FSIS reported that luncheon meats sliced in a retail deli have a 7 fold greater chance of causing listeria infection in consumers than the luncheon meats sliced by the manufacturer (Koo *et al.*, 2013). In a study conducted by Gombas *et al.*, (2003) similar results were also observed. *L. monocytogenes* was found in drastically greater prevalence in deli meat samples sliced by the retailer when compared to deli meat samples sliced in a federally inspected processing plant by the manufacturer. The additional handling and improper storage temperatures may be responsible for the increased

numbers in the retail-sliced samples. Cross-contamination may also occur from cutting boards (both wood and high density polyethylene), stainless steel food-contact surfaces, refrigeration units and workers gloves (including those made of vinyl, latex or polyethylene) (Crandall *et al.*, 2011).

The presence of *L. monocytogenes* on slicers is perpetuated by and dependent on many factors: (i) the attachment properties of the strain, (ii) the biofilm formation properties, (iii) the composition of the food product, (iv) the texture of the food surface in contact with the slicer and (v) the surface condition of stainless steel. To the naked eye, stainless steel surfaces appear smooth and free of crevices. However, microscopic observations revealed the presence of many cracks and areas of corrosion (due to the use of sanitizers). The uneven surface allows for bacteria to more efficiently adhere, forming a niche (Koo *et al.*, 2013; Stone and Zottola, 1985). Deli meat slicers have many removable parts that are connected and sealed with sealers and gaskets. Over time and with heavy use, these parts become worn and degraded creating spaces allowing food debris and moisture to become trapped. These spaces cannot be adequately cleaned allowing pathogenic bacteria to form a niche. The typical problem areas include the ring guard mount, blade guard, and slicer handle (Tarrant, 2014).

In similar studies by Koo *et al.* (2013) and Mertz *et al.* (2014) the microbial diversities of deli meat slicers were analyzed molecularly by the use of denaturing gradient gel electrophoresis (DGGE). Slicers were sampled after their typical cleaning and sanitization processes. Samples were taken from various areas of the slicer that were found in a previous study by Gibson *et al.* (2013) to be most readily cross-contaminated (figure 2) By slicing bologna luncheon meat coated with a fluorescent compound Gibson *et al.* (2013) observed that the (a) cover for the blade sharpener, (b) back plate, (c) blade guard, (d) blade, (e) carriage tray, (f) side wall of the

carriage tray, (g) collection area, (h) side area of collection area and (i) underneath the slicer were most susceptible to contamination and therefore harbor microorganisms. In the study by Mertz *et al*. (2014) the samples were also analyzed for *Escherichia coli*, *Salmonella* and *L. monocytogenes* via specialized growth media. In both studies, pseudomonads, the major causative spoilage bacteria in foods, were the most widely detected bacteria present. In a study involving milk, Marshall and Schmidt (1991), concluded that the proliferation of *L. monocytogenes* was increased in the presence of pseudomonads. The study proposed that pseudomonads provided free amino acids to the environment that allowed for the *L. monocytogenes* to proliferate.

Other bacteria detected in the studies by Mertz *et al.* (2014) and Koo *et al.* (2013) included: *Streptococcus thermophilus, Klebsiella* species*., Paenibacillus* species*., Enterobacter* species, *and Serratia* species. Unlike pseudomonads, lactic acid bacteria have proven to inhibit the growth of *L. monocytogenes* (Piard and Desmazeaud, 1992). Lactic acid bacteria, such as *Streptococcus thermophilus,* have antagonistic properties because of their ability to generate hydrogen peroxide (Price and Lee, 1970). Lactic acid bacteria can drastically decrease the pH of their surrounding environment making it more difficult for other bacteria to proliferate. Lactic acid bacteria may also produce antimicrobial compounds, such as bacteriocins (Klaenammer, 1988; Arihara *et al.*, 1993). *L. monocytogenes* was not detected in either studies by Koo *et al.* (2013) or Mertz *et al.*, (2014) however in a simulated study by Keskinen *et al*. (2008), the biofilm-forming abilities and transfer of *L. monocytogenes* from the slicer blade to the luncheon meats was observed.

Keskinen *et al.* (2008) inoculated stainless steel slicer blades with 6 log CFU/ blade. Exposure times varied (1hr, 6hr and 24hr). After the incubation period, the slicer blades were

cleaned and sanitized. After cleaning and sanitizing, RTE salami and turkey meat was sliced. Consistently, the transfer of *L. monocytogenes* was greater on the first slice than on the second and linearly out to the last slice. This was most likely due to the blades initial exposure to moisture and nutrients from the luncheon meat and to the increased friction. The results of the study suggested that enhanced biofilm-forming abilities are advantageous for *L. monocytogenes* in stressful environments. Significantly greater transfer was seen with the blade inoculated for 6hr rather than the one for 24hr. The overall conclusions of the study reported that the transfer of *L. monocytogenes*, from the blade to the product, was dependent on several factors: time, food product, cell injury and biofilm-forming abilities.

**Cleaning and Disinfection**

Food contact surfaces and processing environments contain water and nutrients to allow for *L. monocytogenes* growth and proliferation. According to FDA regulation, retail luncheon meat slicers should be cleaned and sanitized at least every 4hr of use when used at room temperature (Chavant *et al.,* 2004). If slicers are not sanitized properly or within a timely manner, biofilms will have the opportunity to develop. Ideally, cleaning and sanitization should occur before biofilms develop. Once the biofilm forms, the cells are much harder to eradicate (Lundén *et al.,* 2000).

Sanitizers and disinfectants cannot work effectively to penetrate the biofilm matrix if the surface still has particulate left after an ineffective cleaning process (Simões *et al*., 2010). Before any disinfectant can be properly used, an appropriate cleaning step should be carried out. During cleaning, all debris and residues need to be removed. Mechanical cleaning or clean-in-place (CIP) does not require disassembly. Clean-out-of-place (COP) must be partially

disassembled. Most deli meat slicers need to be manually cleaned, which requires the total disassembly for proper cleaning (Schmidt, 1997).

The use of high temperature and turbulence (from water and scrubbing) have proven effective (Maukonen *et al.*, 2003) in the removal of debris and food particles. To suspend and dissolve food residues, chemical cleaning products typically include surfactants and alkali products to reduce surface tension, emulsify any lipids and disrupt protein structures (Forsythe and Hayes 1998; Maukonen *et al*., 2003). Effective cleaning processes should disrupt the EPS matrix of the biofilm so the sanitizers can have access to the cells within the matrix (Simões *et al*., 2006).

Sanitizers must reduce the microbial load to levels that are considered safe to the consumer. According to the Association of Official Analytical Chemists, an effective sanitizer must reduce the contamination level by 99.999% (5 logs) within 30 sec (Schmidt, 1997). Several antimicrobial products have been shown to effectively control *L. monocytogenes* biofilms. Significant reduction in *L. monocytogenes* has been observed with the use of: chlorine with peracetic acid and perotanoic acid (Fatemi and Frank, 1999), chlorinated-alkali solution (Somers and Wong, 2004), low-phosphate buffer detergent (Somers and Wong, 2004), dual peracid solution (Somers and Wong, 2004), alkaline solution (Somers and Wong, 2004), hypochlorite (Somers and Wong, 2004), chlorine with hydrogen peroxide and ozone (Robbins *et al*., 2005), peroxydes (Pan *et al.*, 2006), quaternary ammonium compounds (QAC) (Pan *et al*., 2006) and chlorine (Pan *et al*., 2006). Studies by Oh and Marshall (1994; 1995) have demonstrated that the use of monolaurin with the use of heat or acetic acid can effectively reduce the presence of *L. monocytogenes* on stainless steel coupons. A study conducted by Crandall *et al*. (2012),

demonstrated that sanitizers commonly used in the deli establishments proved effective in removing 2 to 3 log CFU/ $cm^2$.

Heat has also proven to be an effective form of sanitization (Trivedi *et al*., 2008). Steam allows for a large amount of heat to be transferred during condensation of steam on a food contact surface and in turn rapidly heats the surface (James *et al.*, 2000). At 100˚C, steam has a greater heat capacity than water (James and James, 1997). Steam has the capability to penetrate cracks and crevices that standard cleaning methods cannot (Morgan *et al.*, 1996). In a study by Crandall *et al.* (2011), a 5 log reduction of *L. innocua* was observed when placed in a moist heat oven at 82˚C for 3hr. A dry oven at the same temperature for 15hr proved to be ineffective in reducing the *L. innocua* present. In low-acid canned foods, a 5 log reduction is indication of a sufficient thermal process (Crandall *et al.*, 2011). Although 82˚C for 3 hours in a moist oven proved to be effective, it is not industrially applicable. The high heat/ high humidity conditions would potentially damage the electrical components of the slicer.

Fogging, although there have been limited studies and applications in the food industry, has gained interest recently. Fogging is a method of chemical disinfection that utilizes an automatic spraying device that disperses small droplets of a disinfectant or sanitizer within a closed room (Wirtanen, 1995; Wirtanen and Salo, 2003; Bore and Langsrud, 2005). In a study conducted by Hedrick (1975) chlorine fog was found to significantly reduce the amounts of air-borne microorganisms. In a salmon smoke house study (Bagge-Raven *et al.*, 2003) peracetic acid-based fogging was more effective at microbial reduction than hypochlorite-based foam.

It would be expected that when two methods of sanitation are applied together, their lethal effect on microorganisms would be greater than if one was applied alone; this combination

of treatments is referred to as hurdle technology (Leistner, 2000). In a previous study by Ban *et al.* (2012), steam was used in conjunction with lactic acid. The use of the two treatments together proved to be more potent in killing *L. monocytogenes* than when each treatment was applied separately.

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**Figures**

**Figure 1.** Process of biofilm formation on a deli meat slicer based of the findings of Breyers and Ratner, (2004): : (i) microbes are first pre-conditioned by other macromolecules, (ii) planktonic cells are then deposited from the bulk liquid to the surface, (iii) cells are then adsorbed at the food surface contact surface, (iv) the adsorbed cells are then desorbed from the surface, (v) an irreversible link occurs between the cells, (vi) cell to cell interactions then occur by the production of signaling molecules, (vii) substrates are transported to and within the cell, allowing for replication, growth and extracellular polymeric substrates (EPS) formation (Breyers and Ratner, 2004)

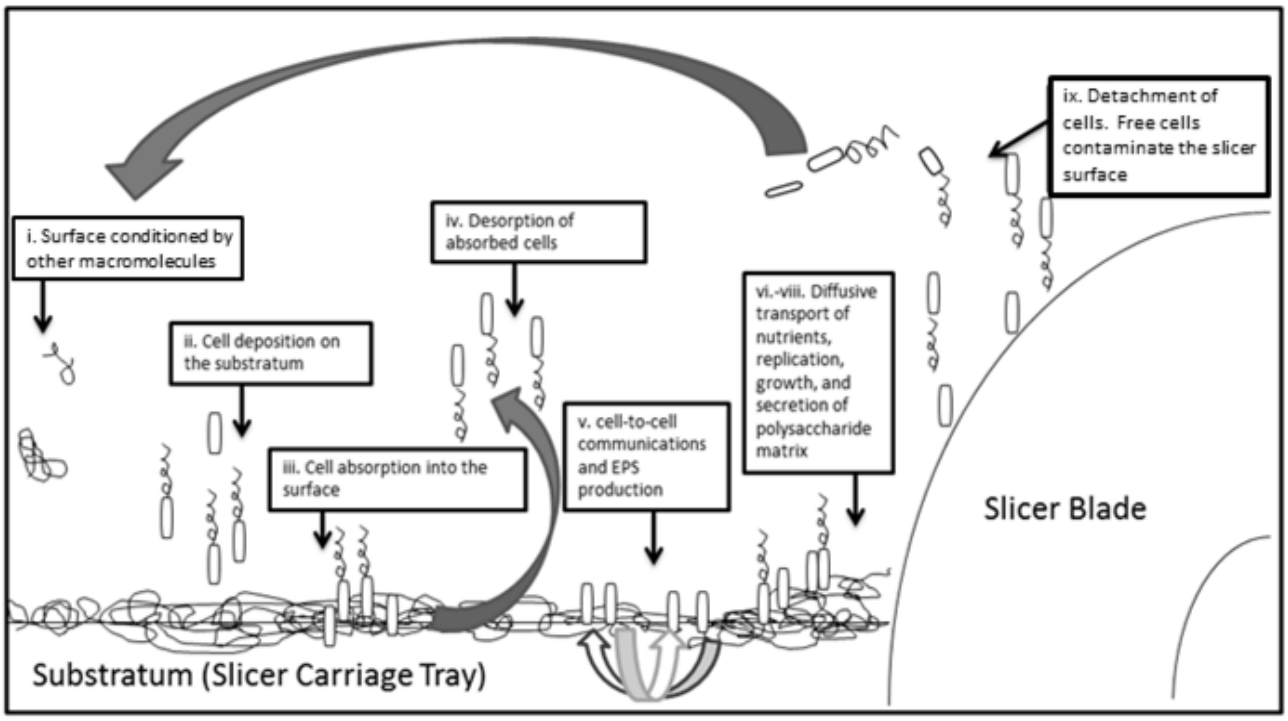
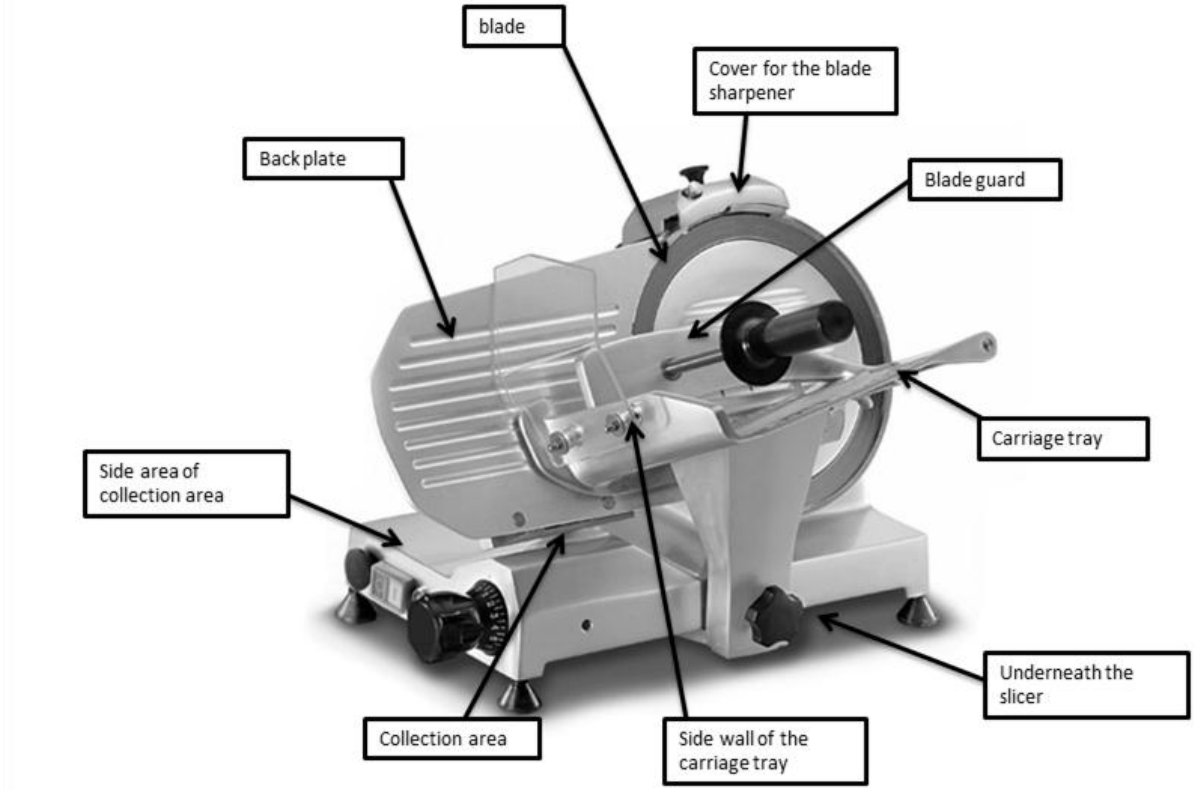


**Figure 2.** By slicing bologna luncheon meat coated with a fluorescent compound Gibson *et al.* (2013) observed that the (a) cover for the blade sharpener, (b) back plate, (c) blade guard, (d) blade, (e) carriage tray, (f) side wall of the carriage tray, (g) collection area, (h) side area of collection area and (i) underneath the slicer were most susceptible to contamination and therefore harbor microorganisms.



Chapter III

# Motility and Biofilm Forming Characteristics of *Listeria* Strains


## Abstract

Within food processing plants, *L. monocytogenes* has frequently been isolated from slicing, dicing, packaging and brining machinery. This machinery has small spaces and narrow openings. These spaces cannot be cleaned adequately, therefore are susceptible to bacterial growth. Planktonic cells form biofilms in order to protect the cell from adverse conditions Once a biofilm is formed, the bacteria are much more difficult to eradicate and can be more resistant to the lethal effects of chlorine .This study analyzes the biofilm forming abilities of different *L. monocytogenes* serotypes and *L. innocua* by observation through motility tests, microtiter plate biofilm assay and microscopy. In order to effectively remove biofilms from food processing equipment, its biofilm forming characteristics need to be analyzed and understood. This study concluded that both flagellated and non-flagellated strains produced biofilms and there was no correlation observed between the production of biofilms and hydrophobicity if the films. The results of this study will provide better understanding of the factors that affect biofilm development on stainless steel and aluminum. This knowledge will help develop more efficient sanitizing methods for food processing equipment.



**Keyword:** *Listeria monocytogenes*, deli slicer, biofilm, motility, hydrophobicity

## Introduction

*L. monocytogenes*, a food-borne pathogen of concern in ready-to-eat foods, is able to survive extreme pH levels that will kill most other food-borne pathogens. It grows well in a pH range of 4.5 to 7.0. No growth is observed at or below 4.0 (Buchanan and Phillips, 1990; Farber and Peterkin, 1991). *L. monocytogenes* can grow between -0.4˚C and 50˚C with an optimum temperature range is between 30 to 37˚C. At 37˚C, growth peaks at 16 to 18 hours of incubation (Gray and Killinger, 1966). *L. monocytogenes* is more heat-resistant than other non-spore forming food-borne pathogens. Through evolutionary changes *Listeria* has developed mechanisms to survive extreme environmental conditions and therefore thrive in food processing plants. Its increased tolerance is attributed to the rising generation of heat shock proteins and the modifications of the fatty acid profile of the cellular membranes (Samelis and Metaxopoulos, 1998).

These are evolutionary modifications made by the bacteria in order to respond to the stress conditions (Samelis and Metaxopoulos, 1998). *L. monocytogenes* has cold stress responses that allow the organism to continue to proliferate at low temperatures. These responses include: (i) changes in the cell membrane structure that maintain lipid fluidity and structural integrity, (ii) cells accumulate cryoprotective osmolytes and peptides to maintain enzyme activity, (iii) alterations occur to the cell's surface proteins that allow access to the environment which offers a greater potential for survival for nutritional reasons, (iv) cells produce "cold shock" proteins that protect against oxidative stress and (v) structural changes occur that maintain the functional and structural stability of ribosomes (which are crucial for protein synthesis) (Bell and Kyriakides 2009). The rising concern of *L. monocytogenes*, as a contaminant in food products, is its ability to survive such extreme conditions.

When *Listeria* cells are under stress and cannot survive, they have the ability to form biofilms for protection. Biofilms are composed of an assembly of microbial cells that are irreversibly linked with an enclosed polysaccharide matrix. The matrix may also contain materials such as lipids and proteins collected from the surface where it forms. The primary function is to protect the bacterial cell from adverse environments and conditions (Breyers and Ratner, 2004). Cells within the biofilm differ from cells in their planktonic form by the genes that are transcribed. Microbes can form biofilms on a variety of surfaces, including natural aquatic systems, drains and drain pipes, living tissues, and food contact surfaces. Planktonic cells attach in the interface between the surface and the bulk aqueous medium. The processes of biofilm formation are not fully understood (Donlan, 2002).

The attachment of cells to food contact surfaces depends on the adhesion surface, the bulk fluid that transports the planktonic cells and the cellular properties. Cells attach more readily to rough textured, hydrophobic surfaces. The roughness of the surface decreases the shear forces and increases the available surface area. Interactions occur between non-polar, hydrophobic (Teflon and plastics) surfaces, the substratum and the cells that allow the cells to overcome repulsion forces (Lundén *et al.*, 2002; Chasseignaux *et al.*, 2002). Food contact surfaces are in constant exposure to liquid media which contain water, carbohydrates, fats, proteins and other nutrients. The aqueous mixture conditions the surface and coats it with polymers which can affect the rate of cellular attachment. A hydrodynamic boundary layer occurs between the substratum and the liquid medium. The thickness of the boundary depends on the linear velocity. As the velocity increases, the boundary will decrease and cells will experience greater turbulence. Higher velocities result in more rapid contact with the food

surface and therefore more rapid attachment. However, if velocities are too high, it will result in the detachment of cells from the surface (Donlan, 2002; Simões *et al.*, 2010).

Based on the cells motility, the attachment of planktonic cells may occur passively or actively. Passive attachment occurs by diffusion, fluid movement and gravity while active attachment is driven by the cell surface (Kumar *et al.* 1998). Active attachment is typically facilitated by flagella on the bacterial cell's surface. *L. monocytogenes* can adhere both passively and actively. When *L. monocytogenes* cells are grown between 20 to 30°C, flagella are present and give the cell's surface a negative charge (Briandet *et al.* 1999). Below 30 ˚C, the *mogR* gene is inhibited by an antirepressor GmaR, allowing for flagellar gene transcription. Inversely, above 37 ˚C, the *mogR* gene represses the transcription of the flagellar gene causing the *L. monocytogenes* cells become non-motile. This means *L. monocytogenes* will develop flagella at room temperatures but not at mammalian body temperatures (Peel *et al*., 1988). The flagella give the cell motility which allows for initial interactions between the cell surface and the substratum.

Initial attachment of the cell to the substratum occurs within 5 to 30 seconds (Mittelman 1998). At first, the attachment is reversible because the interactions and forces between the substratum and bacterial cell are weak. The interactions between the two surfaces involve van der Waals and electrostatic forces as well as hydrophobic interactions. During this stage of attachment, the bacterial cells still maintain Brownian motion and therefore can be easily removed with mild shear force (Sutherland, 2001).

## Materials and Methods

### Culture Preparation

Eight different strains of *Listeria monocytogenes* and two strains of *L. innocua* were obtained from the culture collection of the Center for Food Safety at the University of Arkansas-Fayetteville (Table 1). *Pseudomonas aeruginosa* (ATCC 27853) and *P. fluorescens* (ATCC 13525) strains were used as a positive control for biofilm growth. Stock cultures were revived from frozen (-80˚C) stock cultures maintained in tryptic soy broth containing 0.6% yeast extract (TSBYE; Bacto Becton Dickinson Co., Sparks, MD) and supplemented with 16% glycerol. Frozen stocks were inoculated on Bacto tyrptic soy agar with 0.6% yeast extract (TSAYE; Bacto, Becton Dickinson Co.) slants and incubated at 37˚C for 24h. Using a sterile 10μL inoculating loop, samples were transferred into 10mL of TSBYE and subsequently incubated at 32˚C on a shaker for 24 hr.

### Flagella Evaluation

In order to test for the presence of flagella, 10mL tubes of motility test media (MTM) were prepared with 5% Triphenyltetrzolium Chloride (TTC), which gives bacterial growth a red color, and were autoclaved for sterilization. Each strain was inoculated into MTM+TTC with a sterile inoculating needle and then incubated at 27˚C for 24 hr. Tubes were observed for red colored growth diffusing out from the center stab. Tubes observed with these characteristics are considered positive for motility. Tests were conducted in triplicate.

A negative stain, using 2% aqueous uranyl acetate, was used to observe the flagella. The bacteria were grown, centrifuged and then re-suspended in a phosphate buffer saline. A drop of bacteria were placed on 300 mesh copper grid and allowed to sit a minute. The grid was then

placed under the negative stain, allowed to sit a minute and then removed. Then the grid, with the bacteria, was subsequently placed on top of filter paper. A drop of 2% aqueous uranyl acetate was added to the grid and allowed to rest for 1 to 2 min. The grid was removed and placed face up on the filter paper and allowed to dry. The grid was then viewed on a JEOL JEM 1011 transmission electron microscope 1000x (JEOL USA, Inc., Peabody, MA).

**Microtiter Plate Biofilm Assay**

Modified from the protocol developed by Djordjevic *et al.* (2002), 1 mL of each strain was transferred into 9 mL of fresh TSBYE and incubated at 32˚C for 24h on a shaker. After vortexing, 100 µL of each sample was added to the first 9 wells of a 96 well polyvinyl chloride (PVC) microtiter plate (Becton Dickson Labware, Franklin, NJ). Three wells per row contained 90 µL of sterile TSBYE to serve as negative controls. Serial dilutions to $1x10^{-7}$ were carried out by transferring 10µL from the first row of wells to the next. Each well contained 90 µL of sample or sterile TSBYE. Plates were incubated at 32˚C for both 24h and 48h for each sample.

After incubation, wells were washed 5 times with 150µL of sterile deionized water to remove any loose planktonic cells. Plates were then allowed to air dry for 45 min. Each well was then stained with 150µL of 1% crystal violet in water and allowed to incubate at room temperature for 45 min. The crystal violet was removed from each well and the wells were washed with 150µL of sterile deionized water. Plates were allowed to air dry for 10 to 15 min. At this point the plates were set and could be stored at room temperature for several weeks. One hundred and fifty µL of 95% ethanol was added to each well. Plates were allowed to sit at room temperature for 10 to 15 min allowing the reagent to solubilize the crystal violet. One hundred µL from each well was transferred onto a fresh microtiter plate. Plates were then read on a plate

reader (Bio-tek Synergy HT; Biotech Instruments Inc., Winooski, VT) at 570 nm and 680 nm and the optical density (OD) were recorded. Each test was run in triplicate.

**Cell Surface Hydrophobicity**

Each cells' affinity to the solvent n-hexadecane was determined. Each strain was harvested three times by centrifugation at 7000x g for 5 min and then re-suspended in sterile phosphate-buffer saline (PBS) until an optical density of 1.0±0.2, at an absorbance of 420 nm using a Beckman DU640 spectrophotometer (Beckman Coulter, Inc., Pasadena, CA), was reached and the value was recorded. One milliliter aliquot of n-hexane was added to each 1mL suspension. The samples were then incubated at 30˚C for 10 min. After incubation the samples were vortexed for 60 sec and left standing for 15 min to allow the phases to separate. The OD at 420 nm of the volume that was drawn from the aqueous phase was recorded. Hydrophobicity was calculated with the formula [{OD420(before mixing-OD420 (after mixing)}/the OD420 (before mixing)]*100. This was repeated in triplicate with 3 samples per experiment.

**Statistical Analysis**

Percent hydrophobicity was plotted against the quantified biofilm development to obtain an $R^2$ value to calculate correlation.

**Results**

**Flagella Evaluation**

All strains were tested for motility. After 24hrs at 27˚C, the samples were examined for red colored growth diffusing out from the center stab. Only one strain (Lm 97- serotype 1/2a) was found to not have motility (table 1). It can be inferred that Lm 97 is non-flagellated while

all the other strains are flagellated. To confirm this assumption, Lm 24 (1/2 b) and Lm 97 (1/2 a) were examined under a JEOL transmission electron microscope (Figure 3 to 6). Lm 24 was observed to have several flagella attached to the surface of the cell. It is common for *L. monocytogenes* to have 4 to 6 flagella with smaller flagella branching off. The surface of Lm 24 appeared to be "sticky" and rough. Lm 97, as predicted, did not have any flagella on the cell surface. The surface of the cell was textured differently than Lm 24. Lm 97 was observed to have a smoother surface.

**Microtiter Plate Biofilm Assay**

A microtiter plate biofilm assay was conducted on each strain in order to obtain an indirect quantification of biofilm development. After 24hr the two known biofilm formers, *Pseudomonas aeruginosa* and *P. fluorescens,* exhibited an $OD_{680}$ to $OD_{570}$ of 1.5 to 1.7 respectively (Figure 7). Of the *L. monocytogenes* strains tested, Lm 24 had the most biofilm development after 24hr with an $OD_{680}$ to $OD_{570}$ of 1.5, which is comparable to the positive controls. Prolific growth was also seen in motile serotype 1/2c (sample 98) and in non-motile serotype 1/2a (sample 97) with $OD_{680}$ to $OD_{570}$ of 1.2 and 1.0 respectively. On the microtiter plate, non-motile strains can be differentiated from flagellated strains. Flagellated strains will typically have biofilm formation in a ring around the side of the well. Non-flagellated cells will have biofilm development at the bottom of the well (O'Toole, 2011). The other *L. monocytogenes* strains examined had low biofilm development with $OD_{680}$ to $OD_{570}$ ranging from 0.04 to 0.22 after 24hr. Li 169 and Li 192 (both serotype M1) had $OD_{680}$ to $OD_{570}$ of 1.1 and 0.42 respectively.

After 48hr, *Pseudomonas aeruginosa* $OD_{680}$ to $OD_{570}$ increased to 1.9 and *P. fluorescens,* had biofilm development decreased slightly to an $OD_{680}$ to $OD_{570}$ of 1.5. Although Lm 24 displayed the most biofilm development after 24hr, of the *L. monocytogenes* strains tested, the same did not hold true after 48hr. After 48hr Lm 24 had a decreased value in quantified biofilm development with an $OD_{680}$ to $OD_{570\ of}$ 0.78. Lm 97 also had a decrease with an $OD_{680}$ to $OD_{570}$ of 0.45. Lm 98, on the other hand, showed an increase in biofilm development, after 48hrs, with an $OD_{680}$ to $OD_{570}$ value of 1.4. The other *L. monocytogenes* strains tested still showed little biofilm development with $OD_{680}$ to $OD_{570}$ ranging from 0.11to 0.28. *L. innocua* 169 had decreased biofilm development with an $OD_{680}$ to $OD_{570}$ of 0.28. *L. innocua* 192 had $OD_{680}$ to $OD_{570}$ of 3.0 after 48hr.

**Cell Surface Hydrophobicity**

In previous studies, cellular surface hydrophobicity has been correlated to biofilm development. In order to determine if hydrophobicity is related to biofilm formation, each strains' affinity to a polar solvent was calculated. All strains of bacteria tested had a percent hydrophobicity of less than 40% with the lowest at 5% (Figure 8). In order to determine correlation between biofilm formation and surface hydrophobicity, the $OD_{680}$ to $OD_{570}$ values from the microtiter plate biofilm assay were plotted against the percent hydrophobicity calculated. When the cellular surface hydrophobicity was plotted against the $OD_{680}$ to $OD_{570}$ found after 24hr of biofilm development, an $R^2$ value of 0.027 was determined (Figure 9). This concluded that the quantity of biofilm development is not correlated to the cell surface hydrophobicity. When the same was done for the $OD_{680}$ to $OD_{570}$ values after 48hrs, an $R^2$ of 0.032 was calculated (Figure 10). Again, this concluded that cellular surface hydrophobicity and biofilm formation are not correlated.

**Discussion and Conclusions**

In previous studies conducted by Kim and Frank (1994; 1995), as well as Moltz and Martin (2005), it was determined that the biofilm development varied depending on nutritional conditions. The nutritional conditions were believed to influence the cellular surface properties, including hydrophobicity, and therefore influenced the cell's ability to develop biofilms. In another study, the initial adherence of *L. monocytogenes* to fruit surfaces was found to be correlated to the bacteria's surface hydrophobicity (Ukuku and Fett, 2002). A significant correlation between biofilm development and the adherence capabilities of *L. monocytogenes* on PVC was observed in a study by Takahashi *et al.* (2010). In fact, this study stated that it was one of the primary factors in biofilm development on PVC. There were differences observed in the *L. monocytogenes* ability to adhere to PVC because of differences in the hydrophobicity. However, in other studies using glass as the substratum, this same correlation was not observed. Chae *et al.* (2006) found that the initial adherence on glass was not correlated to the cellular surface hydrophobicity. *L. monocytogenes* attachment to glass was found to be strongly related to the electrostatic attractive forces and not to hydrophobicity. In other studies involving a glass substratum, biofilm formation was dependent on incubation temperatures (Bonavenura *et al.*, 2008). This study investigated the cellular surface hydrophobicity and compared it to the quantified biofilm development.

In order to quantify the biofilm development, each strain was subjected to a microtiter plate biofilm assay. One of the major concerns with using the microtiter biofilm assay as an effective way to quantify biofilm development is that it is an indirect enumeration of biofilm development. This occurs by the adsorption of crystal violet, by the bacterial growth, which is then destained. The stain remaining within teach well was assumed to be adhered to bacterial

growth. The biggest problem with the biofilm assay is that it often times can produce an over estimation of biofilm development. The over estimation is due to the fact that crystal violet is adsorbed by both the planktonic cells and the biofilm growth (Dordjevic *et al.*, 2002). Removing any planktonic cells before staining is critical. Djordjevic *et al*. (2002) studied biofilm development with direct and indirect quantification methods was and determined that although direct quantification was important, there was enough correlation between the two methods that indirectly quantifying the biofilms was a suitable alternative method for rapid detection.

The greatest advantage of using the microtiter plate biofilm assay is that it allows for a rapid analysis of adhesion properties amongst multiple strains at one time. This study demonstrated that both motile and non-motile strains can form biofilms after 24 hr and 48 hr. Also, *L. innocua* can prove to be an important tool in *L. monoctogenes* work. *L. innocua* had more biofilm development after 48 hr than any *L. monocytogenes* strain or positive control. Since *L. innocua* is non-pathogenic and has the ability to development significant amounts of biofilm, it can be used more safely than *L. monocytogenes*.

In this study no correlation was observed between biofilm development and hydrophobicity after 24 hr and 48 hr. Many other studies have investigated the relationship between biofilm development, attachment and hydrophobicity. Chavant *et al.* (2002) concluded that *L. monocytogenes* cells were mostly hydrophilic in nature. This was found to be true in this study as well, with the cellular surface hydrophobicity of all strains at less than 40% hydrophobic. However several studies have concluded that the cell's surface hydrophobicity is constantly changing due to environmental factors, nutrients and age (Vatanyoopaisarn *et al.*, 2000; Chavant *et al.,* 2002). This current study only analyzed the surface hydrophobicity at one

point. Further work will be needed to investigate the surface hydrophobicity of each strain at various stages in the life of the culture life.

In this experiment it was initially thought that if a cell had flagella, than it would develop more biofilm. Harbron and Kent (1988) stated that flagella give the cell motility, therefore allowing for initial cell to surface interactions necessary to attain attachment. It is known that the beginning steps of biofilm development involve the attachment of the bacteria cells to a substratum. From there, the cells are adsorbed at the surface and irreversibly linked (Breyers and Ratner, 2004). Flagella-mediated motility for initial attachment and biofilm formation is necessary for many gram negative bacteria, such as: *Escherichia coli* and *Campylobacter jejuni* (Lemon *et al*., 2007; Pratt and Kolter, 1998; Kalmokoff *et al.*, 2006). In previous studies, a strong correlation between flagellar motility, adherence and biofilm development on stainless steel was observed (Lemon *et al.*, 2007; Gorski *et al.*, 2003). This knowledge led to the hypothesis that flagellated cells would attach to the surface and give the cells more time to develop a biofilm before being rinsed away. The reverse was thought to be true about cells with no flagella; with no flagella, the cell would have more difficulty attaching to a substratum and therefore would be rinsed away before being able to develop the Protectionective layer. This was not found to be necessarily true in this experiment.

*L. monocytogenes* can be split into 13 different serotypes. The virulence of the strains depends on the serotype. Ninety-eight percent of listeric infections linked to humans are involving 4 primary serotypes: 1/2a, 1/2b, 1/2c and 4b; with 4b being linked to the majority of outbreaks (Wiedmann *et al.*, 1997; Kathariou, 2002). These 4 serotypes were the only *L. monocytogenes* strains tested in this experiment. Of the 8 *L. monocytogenes* strains tested, only 1 was found to be non-motile. The serovar was a non-motile 1/2a (Lm 97). Two other of the *L.*

*monocytogenes* strains tested (Lm 190 and Lm 191) were also serotypes 1/2a, however these 2 were motile. In many previous studies, serotype 1/2a has been associated with more than 50% of *L. monocytogenes* isolates that have been found in the environment and recovered from foods (Aarinsalo *et al.*, 2003; Kathariou *et al.*, 2006). *L. monocytogenes* 190 and Lm 191, although motile, produced little to no biofilm development after 24 hr and 48 hr; while Lm 97, however, was non-motile and had significant biofilm development. This disproved the hypothesis that .the presences of flagella play a key role in biofilm development. However, this experiment took place over 24 hr and 48 hr whereas in an retail application the slicing equipment would be cleaned every 4hr use. In further testing, the biofilm development after 2 hr, 4 hr, 6 hr and 8 hr will need to be examined between the motile and non-motile 1/2a serotypes to ensure that motility does not play a factor in biofilm development.

Although flagella assist in the initial attachment of the cell, it is disputed if it is required for biofilm development. This study helped to prove that biofilm development over longer periods of time (minimum of 24 hr) is not influenced by the presence of flagella. This was also found to be true in Djordjevic *et al*. (2002). Vatanyoopaisarn *et al.* (2000) found flagella to be important in the initial attachment of *L. monocytogenes* to stainless steel after a 10hr period. However, he found no differences in attachment between flagellated and non-flagellated cells after 24hr. The results of this study did not confirm that flagella play a role in the initial stages of biofilm development.

The data found in this study indicates that both flagellated and non-flagellated cells can attach to food surfaces over an extended period of time (24 hr and 48 hr). Although flagellated cells have the potential to attach more rapidly, the role of the flagella in attachment is dependent on the strain and growth conditions. It can also be concluded that total biofilm formation is not

dependent on the presence of flagella. Although flagella may assist in attachment for a short period of time, they do not affect the biofilm formation. In this study both motile and non-motile strains produced significant amount of biofilm. Also, *L. innocua* developed biofilms, so it can prove to be an important tool in *L. monocytogenes* work. This study also concluded that there was no correlation between cellular surface hydrophobicity and biofilm development after 24 hr and 48 hr. The results of this study will provide a better understanding on what factors do and do not affect biofilm development. In order to more effectively remove and eradicate biofilm growth, its' formation must first be understood.

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**Figures**

**Figure 3.** Flagella on sample 24, *L. monocytogenes* 1/2b, grown at 27°C for 24h viewed under a JEOL TEM 1000x.

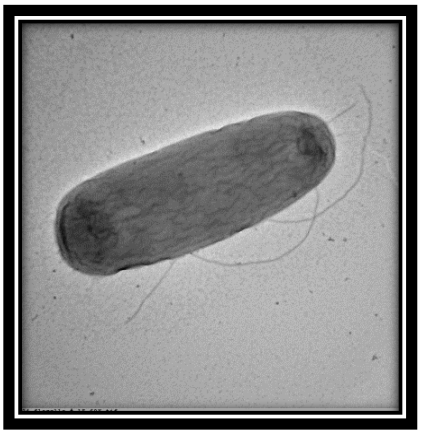


**Figure 4.** . The cellular surface of sample 24, *L. monocytogenes* 1/2b, grown at 27°C for 24h viewed under a JEOL TEM 1000x.

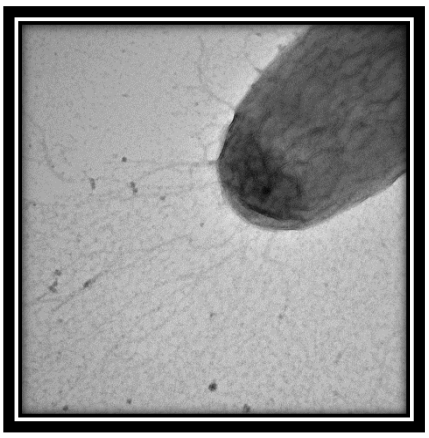


**Figure 5.** The evidence of no flagella on sample 97, *L. monocytogenes* 1/2a, grown at 27°C for 24h viewed under a JEOL TEM 1000x.

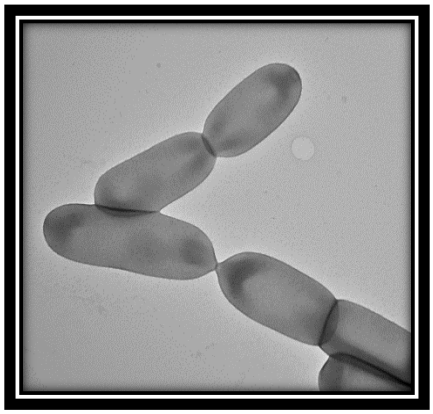


**Figure 6.** The cellular surface of sample 97, *L. monocytogenes* 1/2a, grown at 27°C for 24h viewed under a JEOL TEM 1000x.

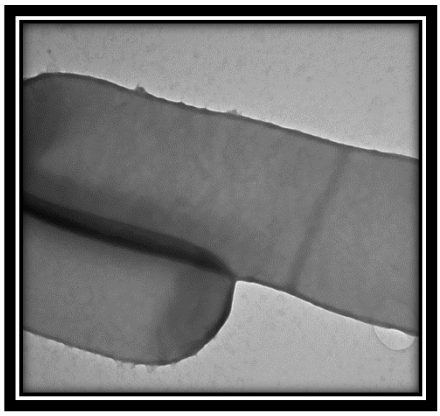


**Figure 7.** Microtiter plate biofilm assay results after 24h and 48h at 32°C and stained with 1% crystal violet solution. Results were read on a plate reader at 570nm and 680nm and the difference is represented in the graph.

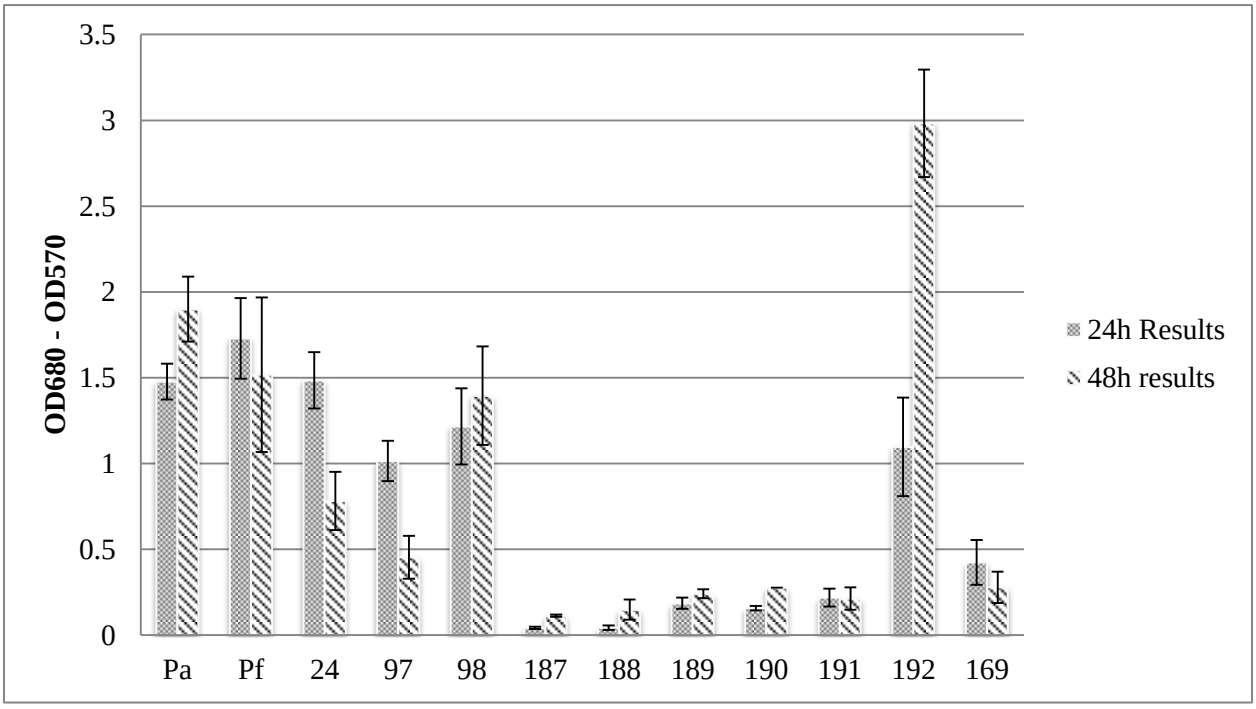


**Figure 8.** Cellular surface hydrophobicity of *Listeria* cells grown at 32°C for 24h was analyzed. Hydrophobicity was conducted by testing each cells' affinity to the solvent n-hexadecane.

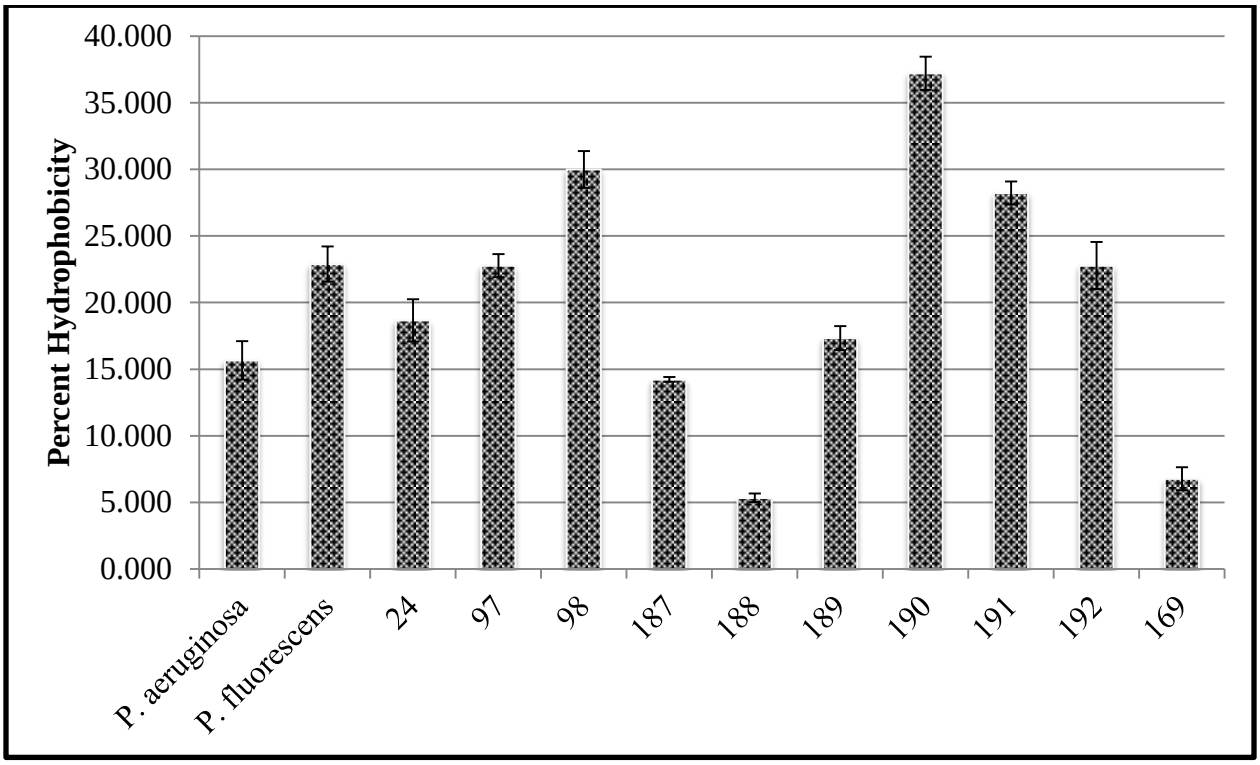


**Figure 9.** The optical densities from the 24hr microtiter plate biofilm assay were plotted against the percent hydrophobicity to observe any correlation. An $R^2$ of 0.0273 was found, so it was determined there was no correlation between biofilm formation and cellular surface hydrophobicity after 24hr.

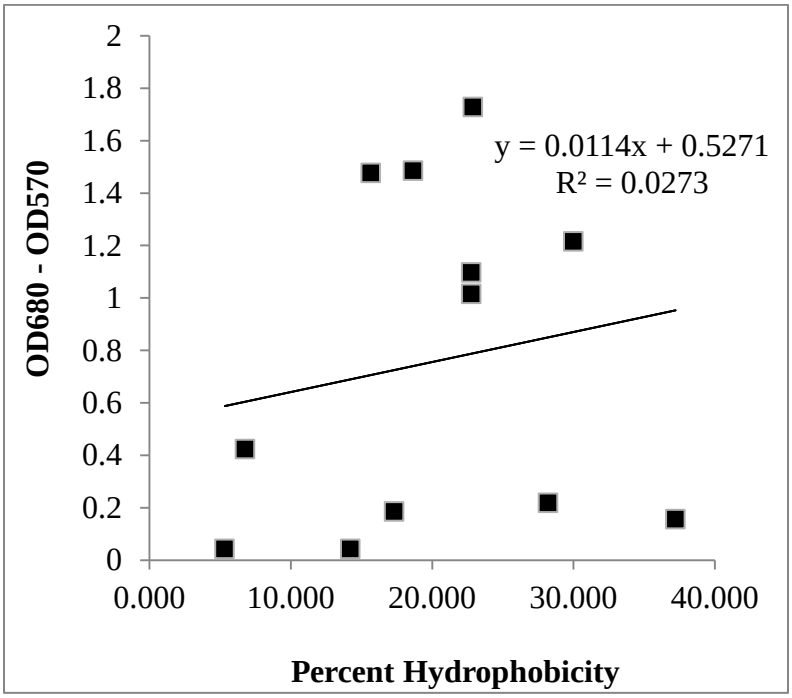
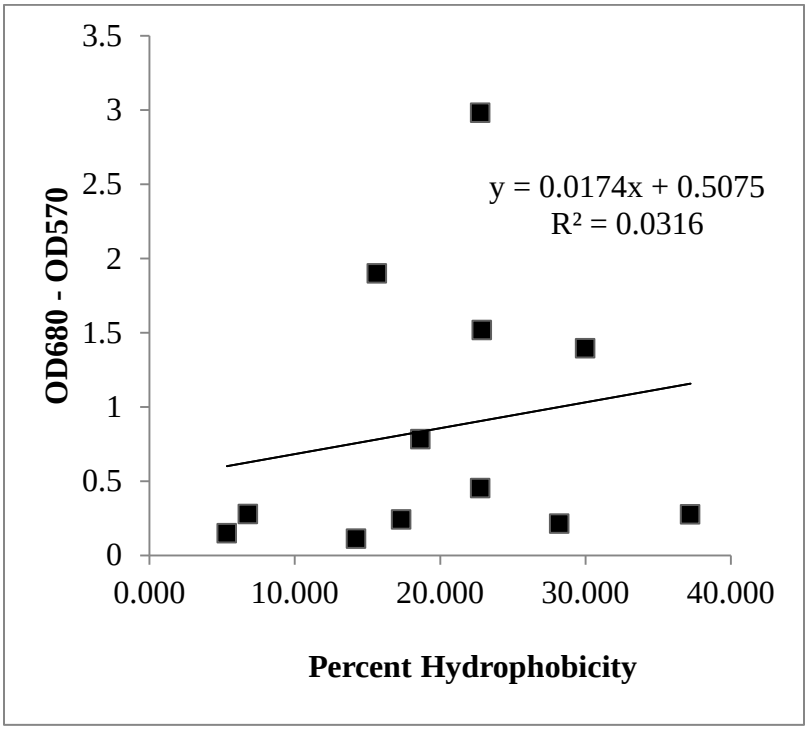


**Figure 10.** The optical densities from the 48hr microtiter plate biofilm assay were plotted against the percent hydrophobicity to observe any correlation. An $R^2$ of 0.0316 was found, so it was determined there was no correlation between biofilm formation and cellular surface hydrophobicity after 48hr.



**Tables**

**Table 1**. *Listeria* strains used in the experiment with their identification numbers as assigned by the Center for Food Safety at the University of Arkansas-Fayetteville, along with serotype and origin (if known). The results of the motility testing are also included.

| Strain | Serotype | Origin | Motile? |
|---|---|---|---|
| **Lm 24** | 1/2 b | | Yes |
| **Lm 97** | 1/2a | | No |
| **Lm 98** | 1/2c | Spinal fluid of a male, Scotland | Yes |
| **Lm 187** | 4b | Cheese outbreak, CDC | Yes |
| **Lm 188** | 4b | | Yes |
| **Lm 189** | 1/2a | Sliced turkey isolate | Yes |
| **Lm 190** | 1/2a | Human illness isolate | Yes |
| **Lm 191** | 1/2a | Human illness isolate | Yes |
| **Li 192** | M1 | | Yes |
| **Li 169** | M1 | Antibiotic resistance to 50 ppm rifampicin and 250 ppm streptomycin | Yes |

# Chapter IV

## The Elimination of *Listeria monocytogenes* Biofilms from Stainless Steel Deli Meat Slicer Components by the use of Hurdle Technologies

**Abstract**

Ready-to-eat (RTE) luncheon sliced in retail delis were found to pose the greatest risk of *Listeria* contamination among all the RTE food assessed in a study conducted by USDA Center for Food Safety and Applied Nutrition and the USDA's Food Safety and Inspection Services (FSIS). A major contributor to this increased risk is because these luncheon meats are sliced in delis. Commercial slicers have many removable parts that are connected with sealer and gaskets, which can become worn over time. These spaces cannot be cleaned adequately, therefore are susceptible to bacterial growth. Effective cleaning processes should disrupt the extra cellular proteins (EPS) matrix of the biofilm, so the sanitizers can have access to the cells within the matrix. Steam allows for a large amount of heat to be transferred during condensation of steam on a food contact surface and in turn rapidly heats the surface. Steam has the capability to penetrate deep into cracks and crevices that standard cleaning methods cannot. This study investigates the synergistic effects of steam and chemical sanitizers on disrupting and removing the biofilms formed on the stainless steel and aluminum coupons cut from deli meat slicer components. Overall there was a 5 to 7 log reduction between the cells recovered from the combined treatments and the cells recovered from the initial inoculation. The sanitizer alone gave a 2 to 3 log reduction and the steam treatment resulted in a 3 to 4 log reduction in cells recovered. The results of this study will provide a better understanding and potential method for the sanitization of industrial deli meat slicers. In turn, the knowledge gained from this study will

reduce the risk of contamination and outbreaks of *L. monocytogenes* and other food-borne pathogens.

**Keywords:** stainless steel, aluminum, *L. monocytogenes*, peracetic acid, chlorine, quaternary ammonia

## Introduction

*L. monocytogenes* is most frequently found to reside on food processing equipment with small spaces and narrow openings, including: slicing, dicing, packaging and brining machinery (Lundén *et al.*, 2002; Autio *et al.*, 1999). Deli meat slicers have proven to harbor *L. monocytogenes* and provide cross contamination. Slicers have many removable parts that are connected and sealed with sealers and gaskets that can become worn and degraded over time and with heavy cleaning chemical use. When these slicer parts become worn, spaces are created allowing food debris and moisture to become trapped. Since these spaces cannot be cleaned adequately bacteria have an opportunity to grow in these niches (Tarrant, 2014). The uneven surface of the stainless steel allows bacteria to more effectively adhere and begin forming biofilms (Koo *et al.*, 2013; Stone and Zottola, 1985). The presence of *L. monocytogenes* on slicers is perpetuated by and dependent on many factors: (i) the ability of the particular strain to attach, (ii) the biofilm formation properties, (iii) the composition of the food product, (iv) the texture of the food surface in contact with the slicer and (v) the surface condition of stainless steel.

Keskinen *et al.* (2008) inoculated stainless steel slicer blades with 6 log CFU/ blade. Exposure times varied (1hr, 6hr and 24hr). After the incubation period, the slicer blades were cleaned and sanitized. After cleaning and sanitizing, RTE salami and turkey meat was sliced. Consistently, the transfer of *L. monocytogenes* was greater on the first first slice than on the second and linearly out to the last slice. This was most likely due to the blades initial exposure to moisture and nutrients from the luncheon meat and to the increased friction. The results of the study suggested that enhanced biofilm-forming abilities are advantageous for *L. monocytogenes* in stressful environments. Significantly greater transfer was seen with the blade inoculated for

6hr rather than the one for 24hr. The overall conclusions of the study reported that the transfer of *L. monocytogenes*, from the blade to the product, was dependent on several factors: time, food product, cell injury and biofilm-forming abilities.

Food-processing equipment, dicers and slicers in particular, manipulate cooked meats and are frequently associated with attached *L. monocytogenes*. Once adhered, it is very difficult to eradicate because adaptive responses have occurred. This allows for recontamination on the processing line (Lundén *et al.*, 2002). Currently the United States Food and Drug Administration (FDA) have established a "zero-tolerance" for the presence of *L. monocytogenes* in a 25 g sample for RTE foods (Czuprynski, 2005). The USDA Center for Food Safety and Applied Nutrition and the USDA's Food Safety and Inspection Services (FSIS), in 2000, conducted an assessment of *L. monocytogenes* contamination in 23 common RTE foods and its risk to public health. Among the RTE foods assessed, deli luncheon meats were found to pose the greatest risk of contamination (FSIS/ USDA, 2003).

Approximately 83% of listeriosis cases contracted from contamination of luncheon meats, can be attributed to deli meats sliced at the retail deli stores (Kause, 2009). In a study by Garrido *et al.* (2009), *L. monocytogenes* was found to be recoverable in 8.5% of samples from meats sliced and packaged by the retail store, while only 2.7% of samples from meats commercially packaged tested positive, indicating the prevalence of *L. monocytogenes* in store sliced deli meats is 3 fold greater than those pre-packaged by the manufacturer. The USDA FSIS reported that luncheon meats sliced in a retail deli have a 7 fold greater chance of causing listeria infection in consumers than the luncheon meats sliced by the manufacturer (Koo *et al.*, 2013). The additional handling and improper storage temperatures may also be responsible for the increased numbers in the retail-sliced samples. Cross-contamination may also occur from

cutting boards (both wood and high density polyethylene), stainless steel food-contact surfaces, refrigeration units and workers gloves (including those made of vinyl, latex or polyethylene) (Crandall *et al.,* 2011).

Sanitizers and disinfectants cannot work effectively to penetrate the biofilm matrix if the surface still has particulate left after an ineffective cleaning process (Simões *et al*., 2010). Before any disinfectant can be properly used, an appropriate cleaning step should be carried out. During cleaning, all debris and residues need to be removed. Mechanical cleaning or clean-in-place (CIP) does not require disassembly. Clean-out-of-place (COP) must be partially disassembled. Most industrial grade deli meat slicers need to be manually cleaned, which requires the total disassembly for proper cleaning (Schmidt, 1997).

To suspend and dissolve food residues, chemical cleaning products typically include surfactants and alkali products to reduce surface tension, emulsify any lipids and disrupt protein structures (Forsythe and Hayes 1998; Maukonen *et al*., 2003). Effective cleaning processes should disrupt the EPS matrix of the biofilm so the sanitizers can have access to the cells within the matrix (Simões *et al*., 2006). Sanitizers must reduce the microbial load to levels that are considered safe to the consumer. According to the Association of Official Analytical Chemists, an effective sanitizer must reduce the contamination level by 99.999% (5 logs) within 30 sec (Schmidt, 1997). The ideal sanitizer should be effective, safe, easy to use, not corrode the surface and be easily rinsed off without leaving any toxic residues. Several antimicrobial products have been shown to effectively control *L. monocytogenes'* biofilms. A study conducted by Crandall *et al*. (2012), demonstrated that sanitizers commonly used in the deli establishments proved effective in removing 2 to 3 log CFU/ $cm^2$.

Heat has also proven to be an effective form of sanitization (Trivedi *et al*., 2008). Steam allows for a large amount of heat to be transferred during condensation of steam on a food contact surface and in turn rapidly heats the surface (James *et al.*, 2000). At 100˚C, steam has a greater heat capacity than water (James and James, 1997). Steam has the capability to penetrate cracks and crevices that standard cleaning methods cannot (Morgan *et al.*, 1996). In a study by Crandall *et al.* (2012), a 5 log reduction of *L. innocua* was observed on coupons made from deli slicers placed in a moist heat oven at 82˚C for 3 hr. A dry oven at the same temperature for 15 hr proved to be ineffective in reducing the *L. innocua* present. Although 82˚C for 3 hr in a moist oven proved to be effective, it is not industrially applicable. The high heat/ high humidity conditions would potentially damage the electrical components of the slicer.

## Materials and Methods

### Coupon Preparation

The stainless steel and aluminum coupons were cut as described in an experiment by Crandall *et al*. (2012) from a used deli Hobart slicer. The coupons were cut into 2x 2.5 cm pieces. The coupons were then washed, wrapped in aluminum foil and autoclaved for 15 min at 121˚C for sterilization prior to inoculation in the experiment.

### Preparation of Cultures from Frozen Culture

The *Listeria* strains chosen were based on our preliminary research done in this study. One of each *L. monocytogenes* strain was chosen for each of the following characteristics: motile, non-biofilm former; motile, biofilm former; non-motile, and non-biofilm former. A strain of *Listeria innocua* was also chosen. A loop full of each frozen *Listeria* strain culture (-20°C) was transferred into individual 9 ml tyrptic soy broth with 0.6% yeast extract (TSBYE;

Becton Dickson Labware, Franklin, NJ) tubes. Tubes were then vortexed to disperse cells in the media. Tubes were incubated overnight at 37°C and the next day each culture was streaked onto PALCAM (Becton Dickson Labware) plates to verify the purity of the cultures. An isolated colony was then picked from the PALCAM plates and inoculated into individual 9 ml TSBYE tubes. Tubes were then vortexed and incubated at 37°C for 24hr.

**Preparation of *Listeria* Strain Cocktail**

One milliliter of each culture (*L. monocytogenes* motile 1/2b, *L. monocytogenes* non-motile 1/2a, *L. monocytogenes* motile 4b and *L. innocua* motile 169) was added into a single 15 ml centrifuge tube to make a cocktail. The cocktail was then vortexed to disperse the cells in the medium and then was centrifuged for 5 min at 5000 rpm. The supernatant was poured off, leaving only the pellet. The cells were re-suspended in sterile 1x phosphate buffered saline (PBS). The initial stock was enumerated by plating serial dilutions onto PALCAM and Plate count agar plates (Becton Dickson Labware). Plates were incubated for 48hr at 37°C.

**Preparation and Inoculation of Coupons**

The stainless steel and aluminum coupons were prepared and 0.1 ml of each culture was inoculated onto them. On the negative control, 0.1 ml of PBS was added. The inocula were spread evenly over the surface with a sterile inoculating loop. The coupons were then placed into petri dishes. The contact time for the cocktail was 4 hr (representative of the time in which slicer parts are disassembled and cleaned according to the FDA ruling.). After 4 hr the sanitizing treatments were applied.

**Preparation of Sanitizers**

Three sanitizers, commonly used within the food industry, were tested: quaternary ammonia (Diversey Inc. Sturtevant, WI), peracetic acid (Decon Labs, King of Prussia, PA) and chlorine (Clorax Company, Oakland, CA). Each sanitizer was tested at use levels lower than their recommended use in order to have sufficient *L. monocytogenes* survivors to have accurate counts. Thus, the quaternary ammonia has a recommended application of 200 ppm but was tested at 5 ppm and 10 ppm. The chlorine has a typical application of 100 ppm but was tested at 10 ppm and 25 ppm. The peracetic acid has a use of 80 to100 ppm but was tested at 10 ppm and 25 ppm. Originally each sanitizer was used according to the manufacturer's instructions for the recommended contact times. However, to ensure countable survivors, the contact times were decreased to 30 sec and then rinsed with sterile deionized water.

**Inactivation of Biofilms via Hurdle Technologies**

In an industrial bread proofer, on proofer mode, 100 ml of sterile water was placed into the pan and evaporated during the heating cycle creating a moist heat environment. The bread proofer was set to 40°C and 47°C for 7hr. In previous studies a temperature of 65°C with steam was found to be the most lethal to bacteria while not affecting the internal mechanisms within the deli slicer (Lindsay *et al*., 2013). In order to ensure survivors, the heat was decreased. The coupons were placed inside the bread proofing oven with thermocouples. Results were logged in a humidity/ temperature datalogger (RHT10; Extech Instruments, Nashua, NH). Two coupons were used per treatment; and each experiment was repeated three times.

Coupons were inoculated and again prepared as above. The coupons were laid out in a BioSafety hood and 1 ml of each sanitizer was applied to individual coupons. Coupons were

rinsed with sterile DI water and allowed to air dry in the biosafety hood. Two coupons were used per treatment; and each experiment was repeated three times.

To determine the efficacy of steam applied with sanitizers (hurdle technology), the coupons were prepared and inoculated as previously described. The sanitizer was applied and then treated with the heat treatment described previously. Two coupons were used per treatment; and each experiment was repeated three times.

**Microbial Sampling After Inactivation**

Modified from the procedure used in Moltz *et al. (*2005), the quantity of bacterial biofilms and cells left on the stainless steel coupons were quantified. Each coupon was placed into 100 mL of sterile peptone and vigorously vortexed for 1 min. Subsequently serial dilutions were conducted- 1:10, 1:100, 1:1000 and 1:10,000 in sterile 0.1% saline solution. This was replicated two times and spread plated on both tyrptic soy agar with 0.6% yeast extract (TSAYE; Becton Dickson Labware) and PALCAM agar. Plates were then incubated at 32˚C for 48hr and the colonies were counted. Each dilution was done in duplicate.

## Results

### Inactivation of Biofilms via Hurdle Technologies

The constant use of sanitizers can corrode the stainless steel and other components of the retail deli slicer. The purpose of this study was to determine if a novel combination of thermal and chemical treatments would have an additive or synergistic effect whereby the combination would be more effective than the singular use of any conventional heat or sanitizer treatment. In previous published studies a moist heat at 65° C has been found to be the optimum heat treatment as a lethal kill step for deli slicers. The steam and temperature combined were the most lethal to bacteria while not affecting the internal mechanisms of the deli slicer (Lindsay *et al*., 2013) For this study, a decreased heat treatment was desired, so in preliminary studies 50°C was the original temperature tested. However there were inconsistently few colonies left after the steam treatment alone. In order to obtain countable data, the moist heat temperatures tested were 40°C and 47°C.

To ensure the temperature were programmed and to record the relative humidity changes within the bread proofer, dataloggers were placed in direct access to the steam (on the unobstructed proofer shelf) and in location with indirect access to the steam (inside the motor compartment of the slicer). We previously determined that the inside of the motor compartment, inside the motor armature would be the "cold-spot". The data was recorded every minute. There were differences in both the relative humidity and temperature when comparing direct and indirect access (figure 11to14). In general, the temperatures recorded from the proofer shelf held steady through the 7 hr run. The temperatures recorded inside the motor compartment fluctuated a bit more. In the motor compartment, the temperatures within the motor compartment rose to

temperatures higher than the surrounding air. Overall, on both the proofer shelf and within the motor compartment, as the relative humidity decreased the temperature increased.

The dataloggers placed on the proofer shelf at 47˚C had a starting relative humidity close to 30% and peaked around at 40% between hour 2 and 3. The final relative humidity recorded was 15% with a temperature of 47.5˚C. The dataloggers placed within the motor compartment at the same temperature, have starting relative humidity values that were more than double. The beginning relative humidity was just over 60% and gradually decreased as the cycle continued. The beginning temperature was approximately 46˚C but had a final temperature of 48.5˚C. The final relative humidity was 30%. The same trends were observed for temperatures around 40˚C.

The cells counts from untreated coupons were: $3.83 \times 10^7$ CFU/cm$^2$ (stainless steel) and $9.37 \times 10^6$ CFU/cm$^2$ (aluminum). Three sanitizers were tested for their efficacy at low concentrations: Quaternary ammonia, peracetic acid and chlorine. Overall, a 2 to 4 log reduction was observed from the application of sanitizers ranging from 5 ppm to 25 ppm (which is approximately a log less than the recommended use level). To compare the difference in the efficacy of sanitizers and the rinsing action of water, coupons were also treated with deionized water only. The coupons treated with water had a 1 to 2 log reduction from the initial counts. In general, the sanitizer treatments were more effective than water. However, it is interesting to note, that the plate counts on the aluminum coupon treated with water alone had lower counts than those on the aluminum coupons treated with quaternary ammonia at 5 ppm and similar counts to chlorine at 10 ppm.

The rinsing step was included to remove any planktonic cells, so only the attached cells remained. The efficacy of the sanitizers was determined by standardizing the results against the

recovered cells from the coupons treated only with deionized water. The percent cells recovered were determined after each sanitizer treatment, with the samples treated only with deionized water as a 100% recovery (figure 15). When comparing each sanitizer at 10 ppm, the samples treated with chlorine had the highest percentage of cells recovered. Peracetic acid and quaternary ammonia preformed similarly.

Coupons treated with 10 ppm quaternary ammonia had 1 log less CFU/ $cm^2$ than the coupons treated with 5 ppm quaternary ammonia. Although, the initial microbial concentrations were not the same on stainless steel and aluminum coupons, the final counts (after the quaternary ammonia treatment with a 30 sec) were similar. Coupons treated with 10 ppm of peracetic acid had a 1 to 3 log reduction while those treated with 25ppm had a 2 to 4 log reduction. When chlorine was used at 10 ppm or 25 ppm a 2 log reduction was achieved on both aluminum and stainless steel coupons. Chlorine preformed least effectively when compared to quaternary ammonia and peracetic acid.

In order to determine the efficacy of the sanitizer treatments combined with the steam treatments, the steam treatments alone had to be tested. Testing the efficacy of the steam treatment alone also will allow for comparison between steam and sanitizer treatments. Most conventional deli slicer cleaning methods emphasize the use of harsh sanitizers. Since the use of sanitizers is emphasized it can be assumed that sanitizers are more effective than steam on the removal of bacteria biofilms. This study did not find that to be true. Coupons exposed directly and indirectly to steam had plate counts 3 to 4 logs less than the untreated coupons. The stainless steel samples (treated at both 40˚C and 47˚C) had a 4 log reduction in microbial concentration when directly exposed to steam and a 3 log reduction when indirectly exposed. The aluminum samples (treated at both 40˚C and 47˚C) had a 3 log reduction when directly

exposed to steam and approximately a 2 log reduction when indirectly exposed to steam. In general, the coupons exposed to direct steam had approximately a log less of bacterial growth than those indirectly exposed.

The efficacy of the moist heat treatments were determined by standardizing the results against the recovered cells from the coupons treated only with deionized water. The percent cells recovered were determined after the 7 hr treatment cycle (figure 16). Overall, a higher percentage of cells were recovered from the aluminum coupons rather than the stainless steel. At 47°C, the percent recovery from the stainless steel coupons with direct exposure to steam was less than 50% while on the aluminum coupons it was over 70%. The stainless steel coupons treated at 40°C with direct exposure to steam had 10% more cells recovered than the stainless steel coupons treated at 47°C with direct exposure.

Based on the previous results, it was assumed that 5 to 8 log reduction would be observed once the treatments were combined (table 2). In the combined treatments, a 5 to 7 log reduction was observed. For all combined treatments, less than a 20% recovery of *Listeria* cells (figure 17 to 18). In general, the coupons treated with higher sanitizer concentrations and exposed directly to steam had the greatest log reductions. More variation was seen in the coupons within the motor compartment. The highest plate counts were observed on the coupons treated with 10 ppm chlorine and located within the motor compartment. At 40°C the stainless steel sample within the motor compartment that was treated with chlorine had the highest percent recovery of cells (approximately 17%). However that same sample treated with chlorine at 25 ppm had 0% recovery of cells. All coupons treated with sanitizers and exposed to a moist heat at 47°C had less than 12% of the bacterial cells recovered. Overall, higher plate counts were observed on

coupons treated with low heat steam and sanitizers at low concentrations than either treatment alone.

**Discussion and Conclusions**

The purpose of this study was to apply a sanitizer as well as a heat treatment in order to reduce the level of each treatment applied. To the naked eye, stainless steel surfaces appear smooth and free of crevices. However, microscopic pictures reveal the presence of many cracks and areas of corrosion. The corrosion is due to general use, the use of sanitizers and abrasion methods used in cleaning. The uneven surface allows for bacteria to more efficiently adhere, forming a niche (Koo *et al.*, 2013; Stone and Zottola, 1985). Industrial slicers have many removal parts to allow for more thorough cleaning. Over time and with heavy use, these parts become worn and degraded creating spaces allowing food debris and moisture to become trapped. These spaces cannot be adequately cleaned allowing pathogenic bacteria to form a niche. The typical problem areas include: ring guard mount, blade guard, and slicer handle (Tarrant, 2014).

An effective sanitizer must reduce the contamination level by 99.999% (5 logs) within 30 sec (Schmidt, 1997). The ideal sanitizer must effective, safe, easy to use, not corrode the surface and be easily rinsed off without leaving any toxic residues. However to be effective enough to be remove biofilms, most sanitizers are also mildly corrosive to processing equipment. In order to decrease the harsh effects of sanitizers and heat, this study decreased both to less lethal doses. This study proved that a low dose of sanitizers alone only achieved a 2 to 4 log reduction. In general as the concentration of the sanitizer doubled, the CFU/$cm^2$ decreased by a log. A study

conducted by Crandall *et al*. (2012), demonstrated that sanitizers commonly used in the deli establishments, at their recommended uses, were effective at removing 2 to 3 log CFU/ $cm^2$.

Interestingly, the action of rinsing with water removed 1 to 2 logs from the coupons. After the coupons were treated with sanitizers, with contact times of 30 sec, they were rinsed. If you subtract the log reductions from the water treatments, then the sanitizers alone were only responsible for 1 to 3 log reduction in cells. Since heat has proven to be an effective form of sanitization (Trivedi *et al*., 2008), it would be hypothesized there would be an increased log reduction.

Although this study did not investigate the use of hot water to rinse the sanitizers from the coupons, it did analyze the use of steam as a heat treatment. Steam allows for a large amount of heat to be transferred during condensation of steam, on a food contact surface, and in turn rapidly heating the surface (James *et al.,* 2000). At 100˚C, steam has a greater heat capacity than water (James and James, 1997). Steam has the capabilities that penetrate cracks and crevices that standard cleaning methods cannot (Morgan *et al.,* 1996), therefore removing bacterial build-up in the hard to reach areas of the slicer. In a study by Crandall *et al.* (2012), a 5 log reduction of *L. innocua* was observed when placed in a moist heat oven at 82˚C for 3 hours. A dry oven at the same temperature for 15 hours proved to be ineffective in reducing the *L. innocua* present. Previous experiments found *L. innocua* to be an effective non-pathogenic substitute in *L. monocytogenes* studies. *L. innocua* exhibited greater biofilm development than the *L. monocytogenes* strains tested. In theory, if a method of treatment has the ability to penetrate and destroy the biofilms produced by *L. innocua*, than the treatment will also be effective on *L. monocytogenes.*

Although 82˚C for 3 hours in a moist oven proved to be effective, it is not industrially applicable. The high heat/ high humidity conditions would potentially damage the electrical components of the slicer. In previous studies the 65°C with steam was found to be the most lethal to bacteria while not affecting the internal mechanisms within the deli slicer (Lindsay *et al*., 2013). Using 65˚C s a starting temperature, the initial temperatures to be tested were 50˚C and 60˚C. After initial testing, all cells were eliminated when treated with steam only at 50˚C. Further testing led this study to use 40˚C and 47˚C when testing the efficacy of the steam treatments.

Overall, steam treatments resulted in a higher reduction of cells than the sanitizers tested. The steam treatments resulted in a 3 to 4 log reduction, for the coupons both directly and indirectly exposed to steam. At both 40˚C and 47˚C a 4 log reduction was observed on the coupons exposed directly to steam. The coupons with indirect steam (representing the hard to reach areas of the slicer) had a 3 log reduction at both 40˚C and 47˚C. Although steam does not reach the 5 log reduction requirement for being an efficient sanitation method, when it is combined with a treatment of a low concentration of sanitizer, it will reach a 5 to 8 log reduction.

At 40˚C in combination with either chlorine, peracetic acid or quaternary ammonia coupons directly exposed to steam had less than a log of survivors. The coupons with direct exposure to steam and treated with sanitizers did not show a significant difference in the log reductions on the stainless steel and the aluminum. In the heat treatments, there was no significant difference between the survivor counts on the stainless steel coupons and the aluminum. However, there was a significant difference observed between the stainless steel and aluminum coupons treated with the chlorine alone and when treated with chlorine and exposed to indirect steam at 40˚C. When the coupons were treated with 25ppm of chlorine, there was more

than a log difference between the growths on stainless steel verses aluminum. Stainless steel exhibited a lower log reduction (2 logs) than the aluminum (3 logs). When the coupons were treated with 10ppm of chlorine and exposed to indirect steam, aluminum had 2 logs greater reduction than stainless steel.

The combined sanitizer and heat treatments with directly exposed to steam had similar results at both 40˚C and 47˚C. The coupons treated within the motor compartment at 47˚C and with sanitizers had a 6 to 7 log reduction, while when the temperature was a adjusted to 40˚C, there was a 5 to 7 log reduction. At 47˚C, with the combined treatments, more variation was observed amongst the samples on the stainless steel coupons than when treated at 40˚C. Although there was more variation, the samples still reached the desired 5 log reduction.

At both 40˚C and 47˚C with direct exposure to steam and treated with sanitizers at concentrations between 5 ppm and 25 ppm, had less than a log of growth (6 to 7 log reduction). Those with indirect exposure, had between none and 2 logs of growth, which still meets the required 5 log reduction requirement for an effective sanitizing method. It can be concluded, that reducing chlorine, quaternary ammonia and peracetic acid concentrations between 10ppm and 25 ppm, while also treating with a moist heat step between 40˚C and 47˚C is an effective sanitation procedure for stainless steel and aluminum components of a deli meat slicer. It is important to note that the thermal treatment took place over 7hr. This sanitation method would not replace the cleaning of the deli slicer every 4 hr required by the FDA Food Code, but would help ensure the removal of the buildup of biofilms.

In a retail setting, at the end of a working period, the slicer should be cleaned. Sanitizers and disinfectants cannot work effectively to penetrate the biofilm matrix if the surface still has

particulate left after an ineffective cleaning process (Simões *et al*., 2010). Before any disinfectant can be properly used, an appropriate cleaning step should be conducted. Most industrial grade slicers need to be manually cleaned, which requires the total disassembly for proper cleaning (Schmidt, 1997). Effective cleaning processes should disrupt the EPS matrix of the biofilm so the sanitizers can have access to the cells within the matrix (Simões *et al*., 2006).

Once the slicer is effectively cleaned, then the sanitizer at a low concentration should be applied. This study found, chlorine, quaternary ammonia and peracetic acid to be effective at achieving a 5 log reduction when used in conjunction with a steam process. However, quaternary ammonia worked more effectively at lower concentrations (5 ppm and 10 ppm) than the other sanitizers tested. Peracetic acid had similar results when used at slightly higher concentrations (10 ppm and 25 ppm). After the recommended contact time by the sanitizer's manufacturer, the deli meat slicer in its entirety, as well as its components, should be placed within the bread proofing oven and 100ml of water should be added to the water pan. In earlier experiments, 100ml was found to give the appropriate volume of moist heat, while ending with an appropriate period of dry heat. The period of dry heat helps to evaporate any moisture in the electrical components of the slicer, so the motor is not damaged during the procedure (Lindsay *et al.*, 2013). The thermal treatment should be applied for 7 hr.

This combination treatment works ideally in the industrial setting at the end of the hours of operation. At the beginning of the next day's hours of operation, any biofilms that may have formed during the shift before will be destroyed by the combination treatments. Further research should be conducted in order to determine the optimum reduction sanitizer concentration and the minimum temperature needed to achieve at least a 5 log reduction. Using lower concentrations and temperatures will decrease the amount of pitting and corrosion on the metal components of a

slicer. With less pitting, there will be fewer crevices from bacteria to adhere and form biofilms. This study presents an improved sanitation method for deli meat slicer components. The results from this study provide better understanding and method for sanitizing industrial grade slicers; therefore reducing the potential for *L. monocytogenes* contamination and outbreaks.

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**Figures**

**Figure 11.** Data collected from the humidity/ temperature datalogger (Extech Instruments, RHT10) after 7 hr at 47˚C with direct exposure to steam.

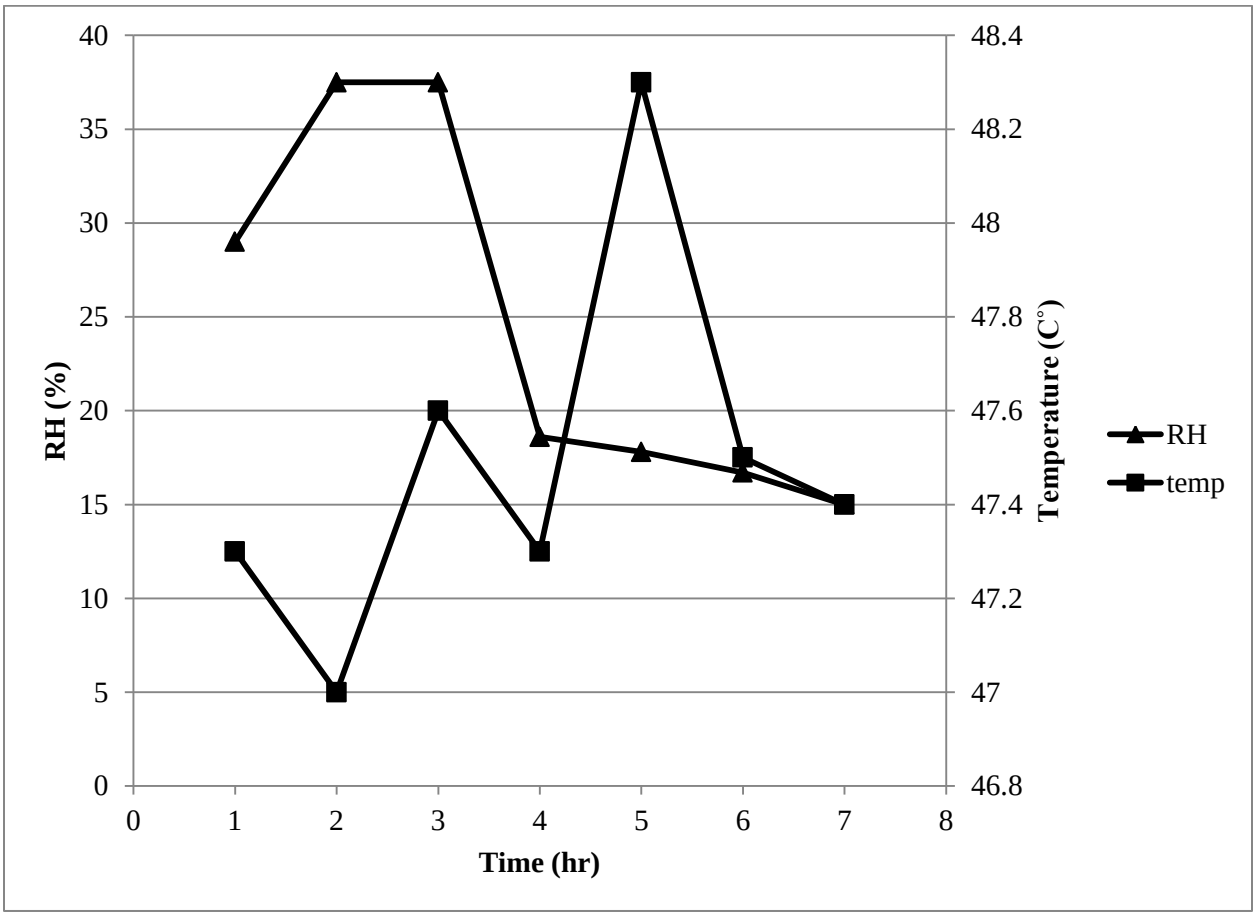


**Figure 12.** Data collected from the humidity/ temperature datalogger (Extech Instruments, RHT10) after 7 hr at 40˚C with direct exposure to steam.

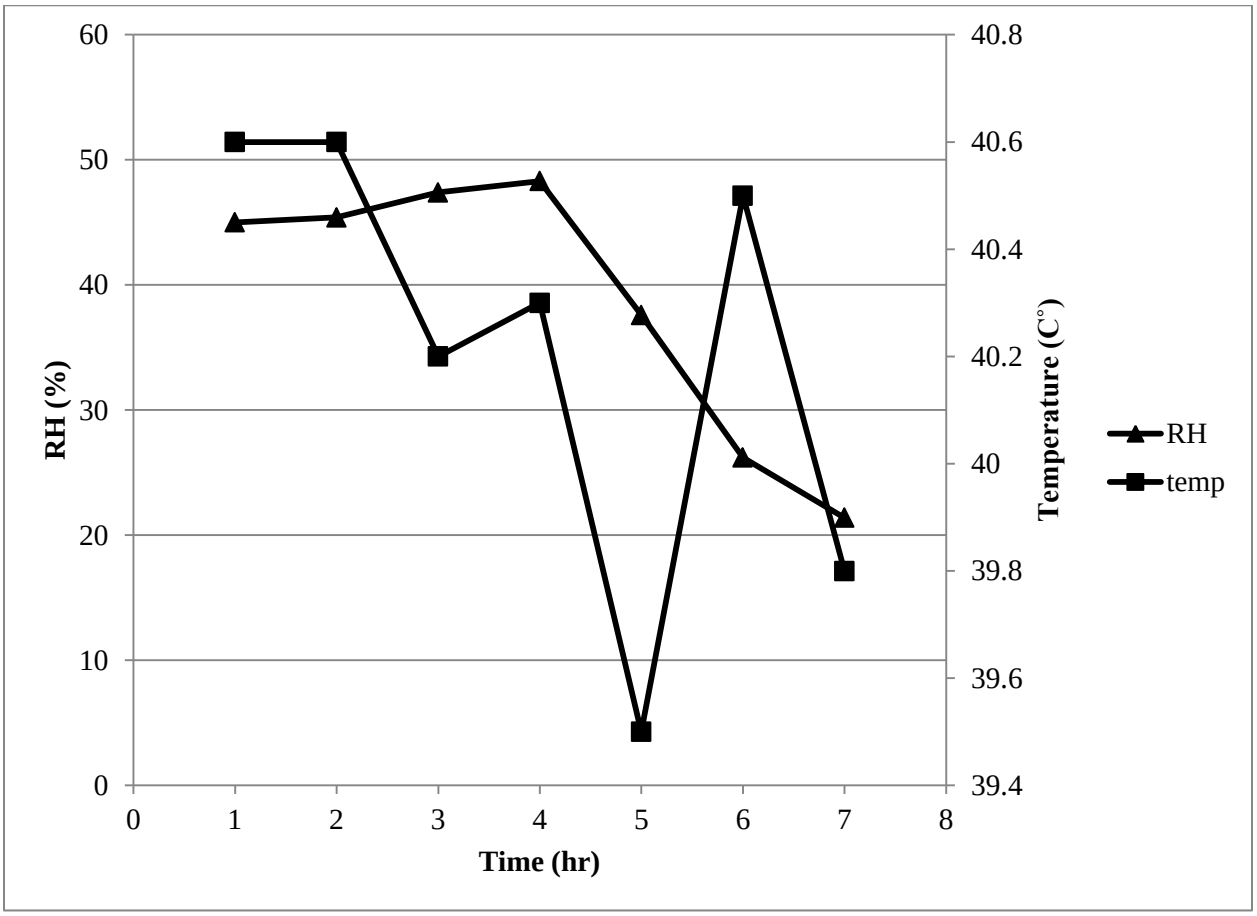


**Figure 13.** Data collected from the humidity/ temperature datalogger (Extech Instruments, RHT10) after 7 hr at 47˚C with indirect exposure to steam.

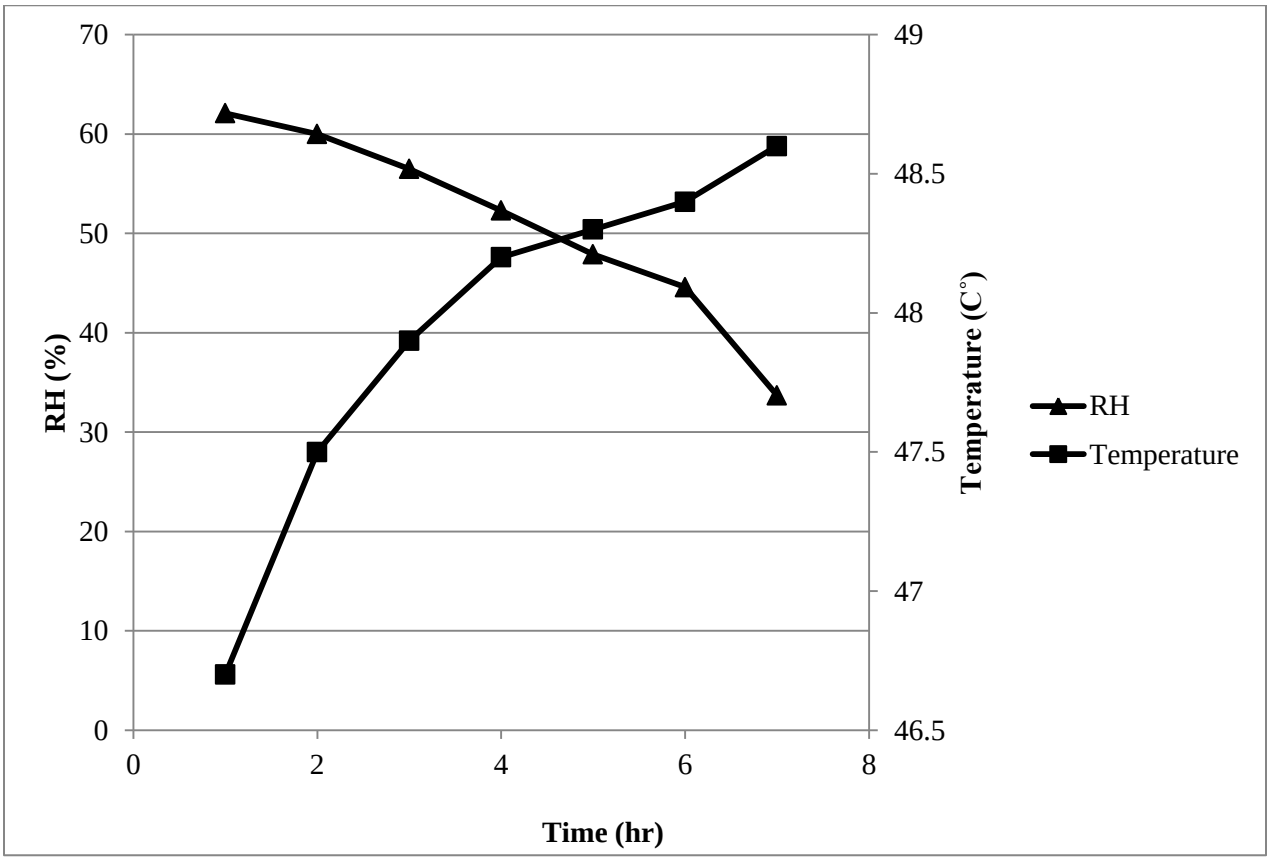


**Figure 14.** Data collected from the humidity/ temperature datalogger (Extech Instruments, RHT10) after 7 hr at 40˚C with indirect exposure to steam.

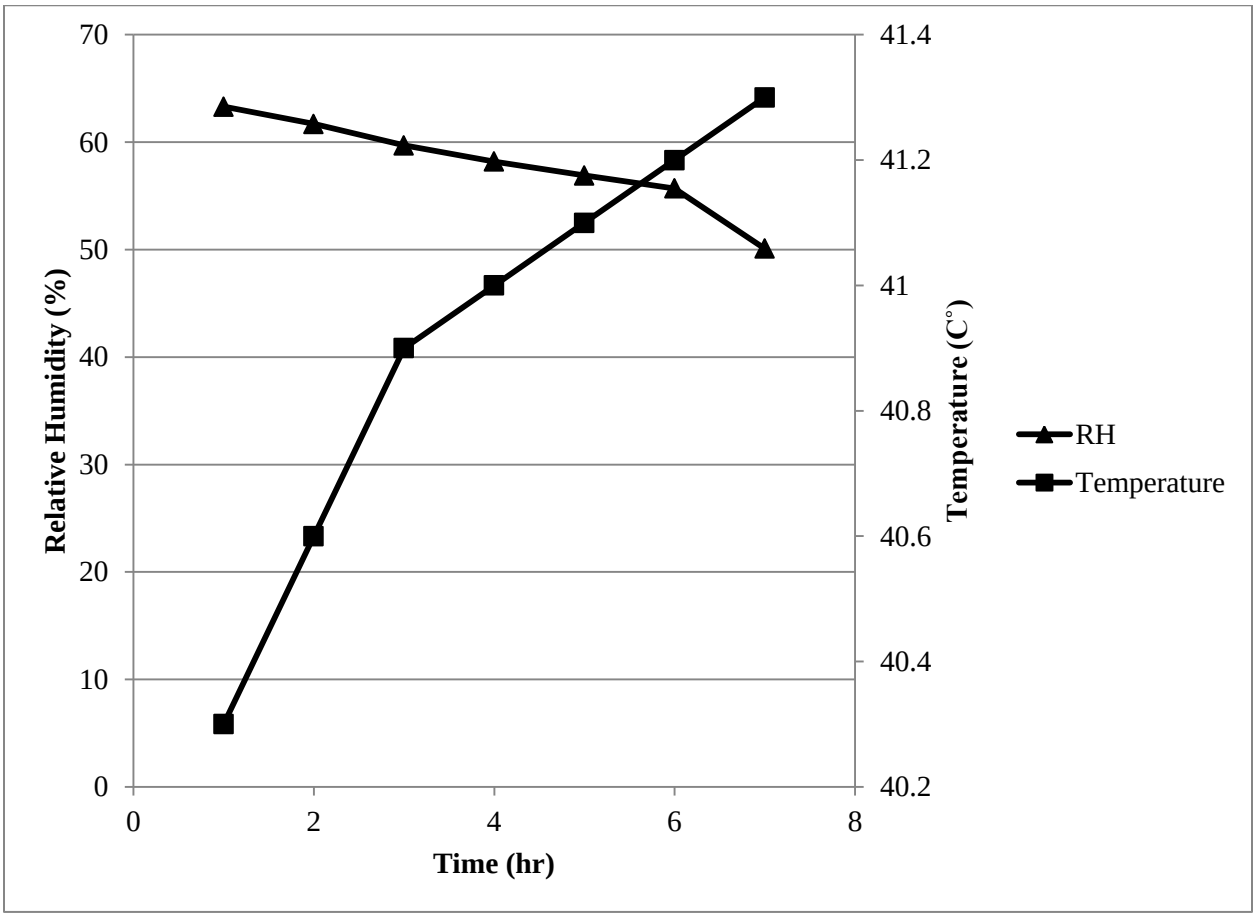
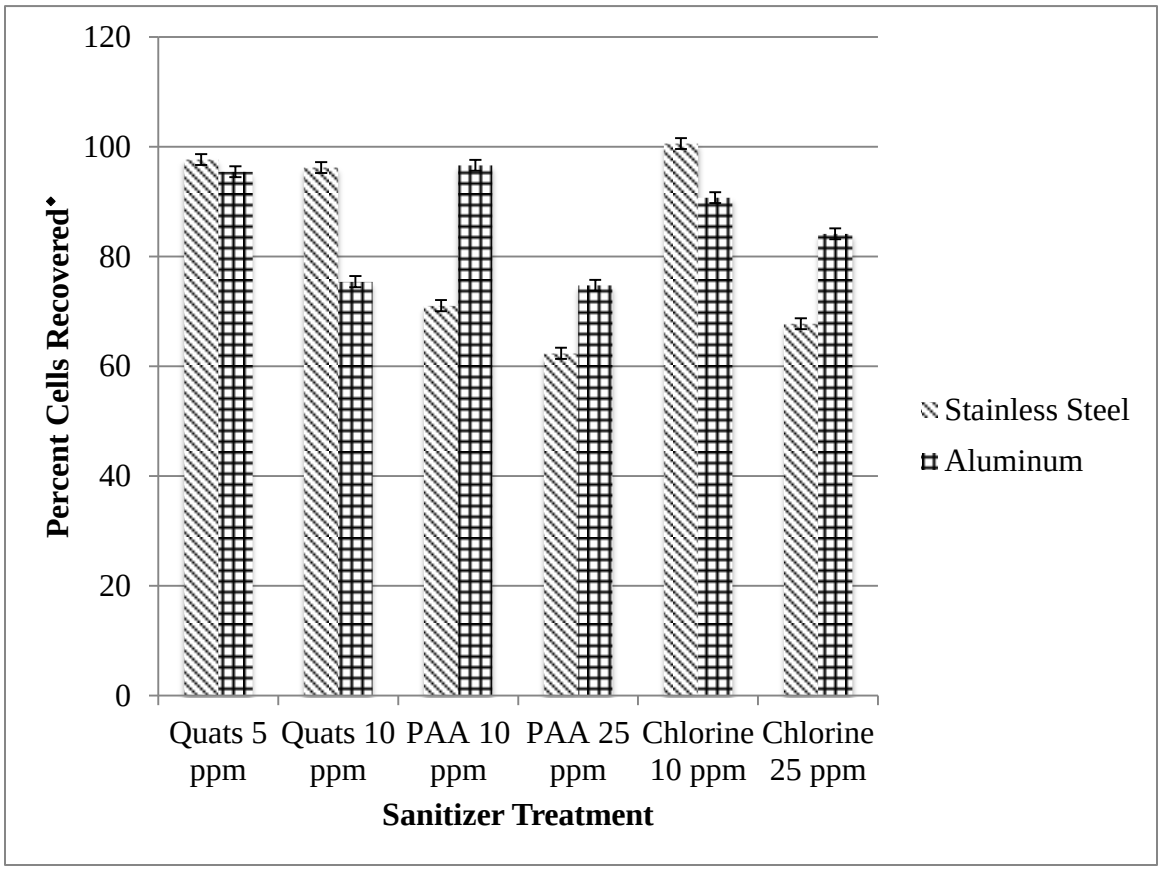
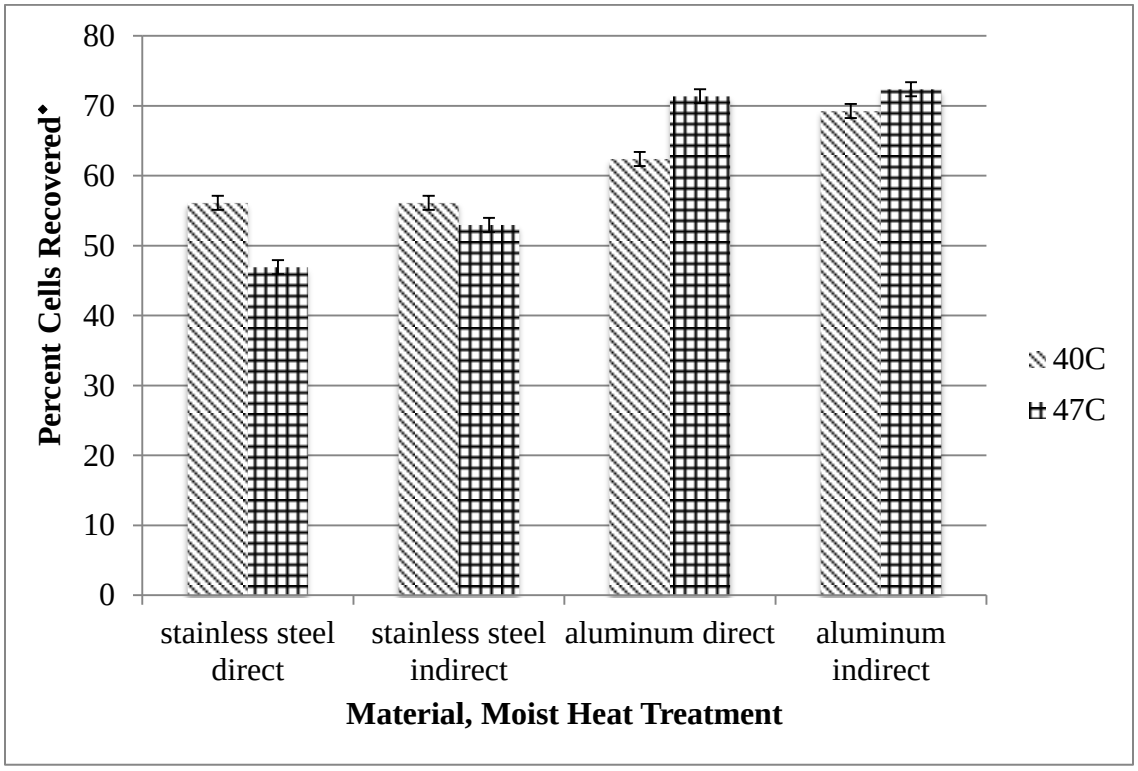


**Figure 15.** Each sanitizer was applied on both stainless steel and aluminum inoculated coupons at 2 concentrations ranging from 5 ppm to 25 ppm with a contact time of 30 sec. Results were recorded in percent cells recovered after treatment.



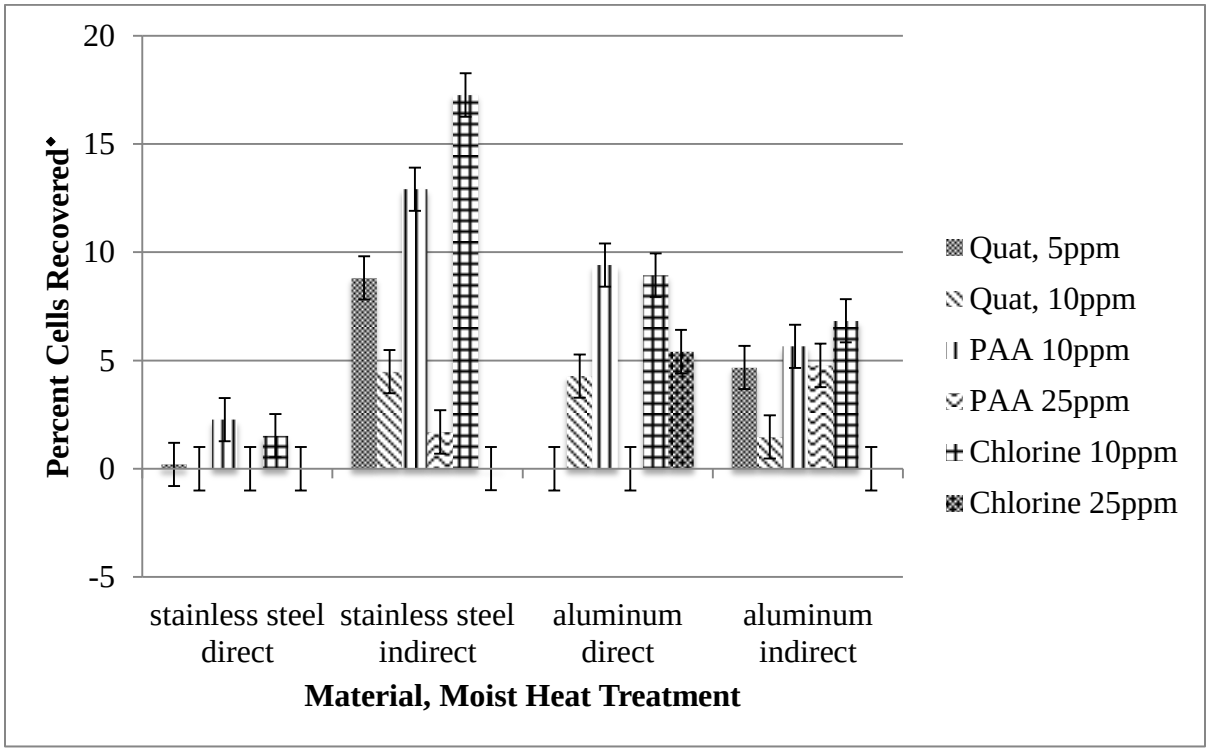

•Percent cells recovered from each coupon was determined by standardinzing the CFU/cm$^2$ recovered after each treatment with the CFU/cm$^2$ recovered from coupons only treated with dionized water.

**Figure 16.** Both stainless steel and aluminum coupons, inoculated with the *Listeria* cocktail, were subjected to a moist thermal treatment for 7hr. Inoculated coupons were subjected to both direct and indirect exposure to steam. Two temperatures were also evaluated (40˚C and 47˚C). Results were recorded in percent cells recovered after treatment.



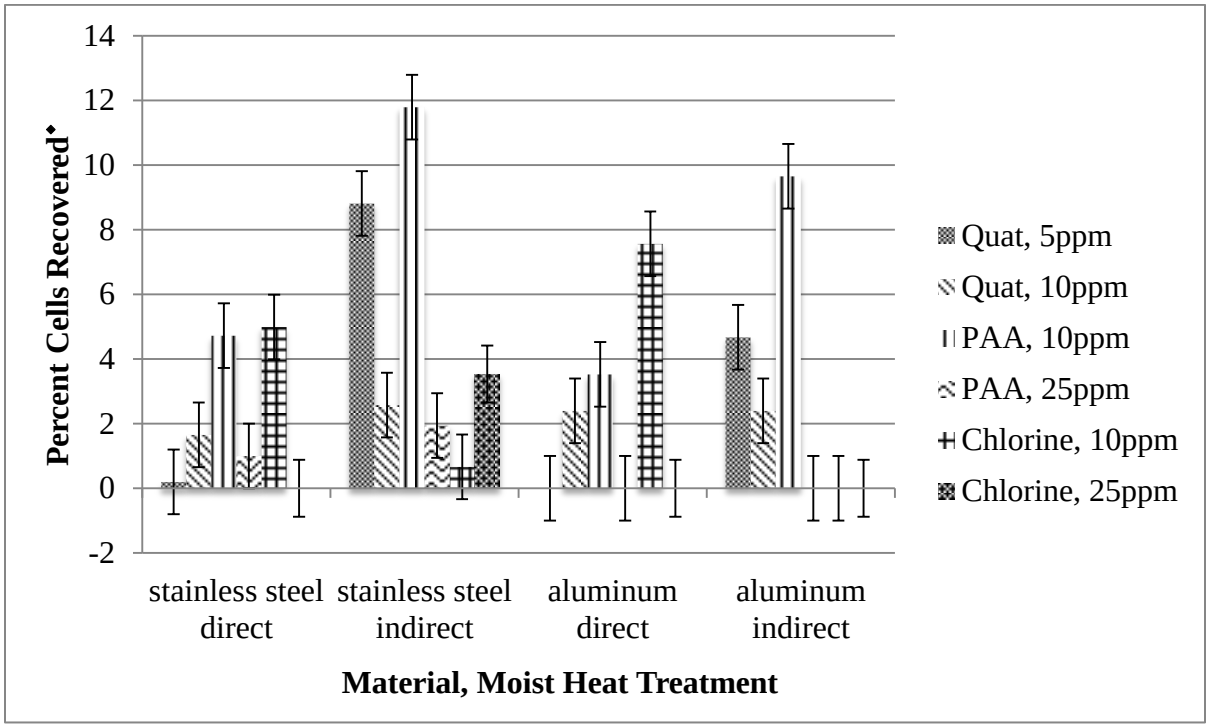

•Percent cells recovered from each coupon was determined by standardinzing the CFU/cm$^2$ recovered after each treatment with the CFU/cm$^2$ recovered from coupons only treated with dionized water.

**Figure 17.** The sanitizer treatments were used in combination with the moist thermal treatment at 40˚C in order to evaluate the efficacy of hurdle technology to eliminate *Listeria* species on stainless steel and aluminum coupons cut from deli meat slicer components. Results were recorded in percent cells recovered after treatment.




⬩Percent cells recovered from each coupon was determined by standardinzing the CFU/cm$^2$ recovered after each treatment with the CFU/cm$^2$ recovered from coupons only treated with dionized water.

**Figure 18.** The sanitizer treatments were used in combination with the moist thermal treatment at 47˚C in order to evaluate the efficacy of hurdle technology to eliminate *Listeria* species on stainless steel and aluminum coupons cut from deli meat slicer components. Results were recorded in percent cells recovered after treatment.




♦Percent cells recovered from each coupon was determined by standardinzing the CFU/cm$^2$ recovered after each treatment with the CFU/cm$^2$ recovered from coupons only treated with dionized water.

**Tables**

**Table 2.** The cells recovered after rinsing the stainless steel and aluminum coupons with deionized water, representing the initial cell concentrations. The table as lists the cells recovered after the application of each sanitizer (30 sec contact time) and temperature treatment cycle (7 hr)

| **Treatment** | | | **Material** | **Cells Recovered After Treatment Time (log[CFU/cm^2])** | |
|---|---|---|---|---|---|
| **Sanitizer** | | **Concentration (ppm)** | | **0 sec** | **30 sec** |
| | Quaternary Ammonia | 5 | Stainless Steel | 5.87 ± 0.18 | 5.90 ± 0.09 |
| | | 5 | Aluminum | 5.57 ± 0.08 | 5.31 ± 0.11 |
| | | 10 | Stainless Steel | 5.87 ± 0.18 | 3.98 ± 0.08 |
| | | 10 | Aluminum | 5.57 ± 0.08 | 4.20 ± 0.21 |
| | Peracetic Acid | 10 | Stainless Steel | 5.87 ± 0.18 | 4.17 ± 0.04 |
| | | 10 | Aluminum | 5.57 ± 0.08 | 5.38 ± 0.18 |
| | | 25 | Stainless Steel | 5.87 ± 0.18 | 3.66 ± 0.03 |
| | | 25 | Aluminum | 5.57 ± 0.08 | 4.16 ± 0.20 |
| | Chlorine | 10 | Stainless Steel | 5.87 ± 0.18 | 5.73 ± 0.04 |
| | | 10 | Aluminum | 5.57 ± 0.08 | 5.05 ± 0.11 |
| | | 25 | Stainless Steel | 5.87 ± 0.18 | 5.65 ± 0.05 |
| | | 25 | Aluminum | 5.57 ± 0.08 | 4.69 ± 0.13 |
| **Moist Heat** | | **Temperature (°C)** | | **0 hr** | **7 hr** |
| | Direct | 40 | Stainless Steel | 5.87 ± 0.18 | 3.29 ± 0.05 |
| | | 40 | Aluminum | 5.57 ± 0.08 | 0.90 ± 0.68 |
| | | 47 | Stainless Steel | 5.87 ± 0.18 | 0.29 ± 0.59 |
| | | 47 | Aluminum | 5.57 ± 0.08 | 0.37 ± 0.53 |
| | Indirect | 40 | Stainless Steel | 5.87 ± 0.18 | 0.77 ± 0.75 |
| | | 40 | Aluminum | 5.57 ± 0.08 | 0.64 ± 0.72 |
| | | 47 | Stainless Steel | 5.87 ± 0.18 | 0.77 ± 0.75 |
| | | 47 | Aluminum | 5.57 ± 0.08 | 0.73 ± 0.79 |

## GENERAL CONCLUSIONS

The purpose of this study was to observe *L. monocytogenes* biofilm development and its relationship with motility and cellular surface hydrophobicity. It was hypothesized that flagella play a critical role in biofilm formation, and that there was a correlation between cellular surface hydrophobicity and biofilm development. The data presented here disproved the hypotheses. The results in this study indicated that both flagellated and non-flagellated cells can attach to food surfaces over an extended period of time. Although flagellated cells have the potential to attach more rapidly, the role of the flagella in attachment is dependent on the strain and growth conditions. In this study both motile and non-motile strains produced significant amounts of biofilm. Also, *L. innocua* developed biofilms, so it can prove to be an important tool in *L. monocytogenes* work. This study also concluded that there was no correlation between cellular surface hydrophobicity and biofilm development after. Further work should investigate shorter contact times to evaluate if flagella play a role in the initial stages of biofilm development.

The second objective of this study was to use low concentrations of sanitizers in combination with a moist, low temperature thermal treatment. All treatments applied achieved the 5 log reduction from the initial microbial concentration. It was also concluded that the moist thermal treatment proved to be more effective at removing *L. monoctogenes* from the stainless steel and aluminum deli slicer components than the sanitizer treatments.

The combination treatment is ideal in the industrial setting at the end of the hours of operation. At the beginning of the next day's hours of operation, any biofilms that may have formed during the shift before will be destroyed by the combination treatments. Further research should be conducted in order to determine the optimum reduction sanitizer concentration and the

minimum temperature needed to achieve at least a 5 log reduction. Using lower concentrations and temperatures will decrease the amount of pitting and corrosion on the metal components of a slicer. With less pitting, there will be fewer crevices from bacteria to adhere and form biofilms. This study presents an improved sanitation method for deli meat slicer components. The results from this study provide better understanding and method for sanitizing industrial grade slicers; therefore reducing the potential for *L. monocytogenes* contamination and outbreaks.

Appendix I

**Research Compliance Protocol Letter**

IBC#: 08028

**Please check the boxes for each of the forms that are applicable to the research project you are registering. The General Information Form - FORM 1 (this form) MUST be completed on all submitted project registrations, regardless of the type of research.**

- ☐ Recombinant DNA (EVEN IF IT IS EXEMPT from the NIH Guidelines.) (FORM 2)
- ☑ Pathogens (human/animal/plant) (FORM 3)
- ☐ Biotoxins (FORM 4)
- ☐ Human materials/nonhuman primate materials (FORM 5)
- ☐ Animals or animal tissues and any of the above categories; transgenic animals or tissues; wild vertebrates or tissues (FORM 6)
- ☐ Plants, plant tissues, or seed and any of the above categories; transgenic plants, plant tissues, or seeds (FORM 7)
- ☐ CDC regulated select agents (FORM 8)

**To initiate the review process, you must attach and send all completed registration forms via email to ibc@uark.edu. All registration forms must be submitted electronically. To complete the registration, print page 1 of this form, PI sign, date, and mail to: Compliance Coordinator-IBC, 120 Ozark Hall, Fayetteville, AR 72701, or FAX it to 479-575-3846.**

**As Principal Investigator:**

- ☑ I attest that the information in the registration is accurate and complete and I will submit changes to the Institutional Biosafety Committee (IBC) in a timely manner.
- ☑ I am familiar with and agree to abide by the current, applicable guidelines and regulations governing my research, including, but not limited to: the NIH Guidelines for Research Involving Recombinant DNA Molecules and the Biosafety in Microbiological and Biomedical Laboratories manual.
- ☑ I agree to accept responsibility for training all laboratory and animal care personnel involved in this research on potential biohazards, relevant biosafety practices, techniques, and emergency procedures.
- ☑ If applicable, I have carefully reviewed the NIH Guidelines and accept the responsibilities described therein for principal investigators (Section IV-B-7).
- ☑ I will submit a written report to the IBC and to the Office of Recombinant DNA Activities at NIH (if applicable) concerning: any research related accident, exposure incident, or release of rDNA materials to the environment; problems implementing biological and physical containment procedures; or violations of NIH Guidelines.
- ☑ I agree that no work will be initiated prior to project approval by the IBC.
- ☑ I will submit my annual progress report to the IBC in a timely fashion.

**Principal Investigator Typed/Printed Name:** Dr. Philip G. Crandall

**Signature (PI): _____ Date: _____**

**CONTACT INFORMATION:**

**Principal Investigator:**

| | |
|---|---|
| Name: | Philip G. Crandall |
| Department: | Food Science |
| Title: | Professor |
| Campus Address**:** | FDSC N213 |
| Telephone: | 479-575-7686 |
| *After Hours Phone: | 479-442-9973 |
| Fax: | 479-575-6936 |
| E-Mail: | crandal@uark.edu |

**Co-Principal Investigator:**

| | |
|---|---|
| Name: | Steven C. Ricke |
| Department: | Food Science |
| Title: | Donald "Buddy" Wray Chair in Food Safety and Director of the Center for Food Safety in the Institute of Food Science and Engineering |
| Campus Address**:** | FDSC E-27 |
| Telephone: | 479-575-4678 |
| *After Hours Phone: | 479-387-4433 |
| Fax: | 479-575-6936 |
| E-Mail: | sricke@uark.edu |

***Required if research is at Biosafety Level 2 or higher**

**PROJECT INFORMATION:**

**Have you registered ANY project previously with the IBC?** Choose an item.

**Is this a new project or a renewal?**

○ New Project ◉ Renewal

| | |
|---|---|
| Project Title: | Cost Effective Treatments to Minimize Listeria monocytogenes Cross Contamination of Ready-To-Eat Meats by the In-Store Deli Meat Slicer |
| Project Start Date: | 7/1/2008 |
| Project End Date: | 6/30/2014 |
| Granting Agency: | American Meat Institute Foundation and the National Integrated Food Safety Initiative |

**Indicate the containment conditions you propose to use (check all that apply):**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ☑ Biosafety Level 1 | Ref: 1 2 | ☐ Biosafety Level 1A | Ref: 1 2 | ☐ Biosafety Level 1P | Ref: 1 2 |
| ☑ Biosafety Level 2 | Ref: 1 2 | ☐ Biosafety Level 2A | Ref: 1 2 | ☐ Biosafety Level 2P | Ref: 1 2 |

☐ Biosafety Level 3 Ref: 2 ☐ Biosafety Level 3A Ref: 2 ☐ Biosafety Level 3P Ref: 2

References:


1: Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition
2: NIH Guidelines for Research Involving Recombinant DNA Molecules
3: University of Arkansas Biological Safety Manual


**If you are working at Biosafety Level 2 or higher, has your laboratory received an onsite inspection by the Biosafety Officer or a member of the IBC?**

◉ Yes ○ No

**If yes, enter date if known:** 1/1/2011
**If no, schedule an inspection with the Biological Safety Officer.**

**Please provide the following information on the research project (DO NOT attach or insert entire grant proposals unless it is a Research Support & Sponsored Programs proposal).**

**Project Abstract:**


Significant advances have been made by the meat and poultry industries to minimize environmental contamination of ready to eat (RTE) sliced deli meats using improved sanitation and antimicrobials that suppress the outgrowth of low levels of Listeria monocytogenes (Lm). The next step is research focused on more effective cleaning and sanitizing of the deli slicer to further reduce the risk of listeriosis. At the completion of this research, we anticipate that meat companies and their customers who operate delis will have additional Best Practices based on new data that demonstrate a significant reduction in Listeria monocytogenes on the deli meat slicer. This research can reduce the cross-contamination of Lm on RTE luncheon meats. The research will increase consumers' desires for the convenience of RTE foods by increasing their confidence that RTE deli meats are safe for their families.


**Specific Aims:**

**1) Measure the effectiveness of current deli operators' recommended cleaning and sanitation practices in removing Listeria and Listeria biofilms 2) Assess the effectiveness of "hot boxes" to sanitize clean slicers overnight for complete destruction of Listeria in biofilms on food contact surfaces 3) Effectiveness of various types of cleaning cloths on removal of contaminants from surfaces commonly found in delis.**

**Relevant Materials and Methods (this information should be specific to the research project being registered and should highlight any procedures that involve biohazardous or recombinant materials):**

**a.** Preparation of L. monocytogenes cultures. All vortexing, pipetting and inoculations of media with L. monocytogenes will be performed within a biological safety cabinet. A cryogenic vial

containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37°C or 42°C). These cultures will be used for the following procedures:

**b.** L. monocytogenes biofilm formation upon deli slicer material. Cultures will be incubated at 37 °C for 24 h. One ml of the 24 hour culture will be transferred to 10 ml of modified Welshimer's broth (MWB). After 24 hours a second passage into MWB will be made to build inoculum to approximately 108-9 CFU/ml. After the final incubation period cultures will be centrifuged individually in sealed screw capped centrifuge tubes in a sealed centrifuge at 4000 g for 15 min and resuspended in phosphate buffer solution. Each culture will be serially diluted and plated onto TSA+YE agar to determine inoculum levels. This inoculum will be serially diluted to give an approximate 10-3 CFU/ml inoculum. Square coupons measuring 20 X 15 cm2 will be cut from deli slicer components (table, back plate, blade guard, blade, and collection area). Deli slicer components will be purchased for this project and any uncut remaining pieces, cut and used pieces will be destroyed after the experiments are completed. Coupons made of newly purchased stainless steel and cast aluminum will also be used. The stainless steel coupons will be made corrosive resistant by immersing them in 25% nitric acid for 8 h. Newly purchased coupons and coupons cut from the deli slicer components will be cleaned by soaking in Alconox detergent solution (prepared by instructions) with agitation, or by sonication. Coupons will then be rinsed three times with deionized water, and autoclaved at 121 °C for 15 min. Sterile coupons will be aseptically placed in sterile 6 well tissue culture plates containing sterile #1 Whatman filter paper, slightly moistened with sterile deionized water (to keep at 100% humidity). A 0.1 ml 12 h inoculum of L. monocytogenes will be pipetted onto each sterile coupon and spread evenly with a disposable inoculation loop. Petri dishes will be placed at 20 °C for 3 h. To remove planktonic cells, the coupons will be washed carefully by rinsing with 20 ml of sterile potassium phosphate buffer (PPB – 50 mM, pH 7.0). Afterwards, 0.1 ml of sterile TSB will be added to each coupon and placed in incubator at 37 °C for 24 h. Medium will be added daily for 4 days.

**c**. Effectiveness of santizers in removing biofilms. Coupons with L. monocytogenes biofilm will be washed with sterile saline three times and set up in groups for each sanitizer. Three replicates will be run per group. Each group will be subjected to the test sanitizer (diluted per the manufacturer's directions). Samples will be treated for 60 s or per manufacturer's recommendations. After set time, the solutions (on the coupons) will be neutralized with lecithin buffer solution. Biofilm will be removed from each coupon using sterile calcium alginate fiber-tipped swabs (no. 14-959-82, Fisher Scientific), soaked in sterile 0.1% peptone water. Coupons will be swabbed three times and test swab tips will be placed in plastic screw-tap tubes (50 ml, Corning), with 20 ml of 1.0% sodium citrate, vortexed and plated on TSA+YE agar. Plates will be incubated for 48 h at 37 °C, and enumerated.

**d.** Effectivenss of "dry heat" for sanitizing deli slicer materials. Coupons with L. monocytogenes biofilms will be sealed into heat resistant plastic bags. A single side adhesive foam pad will be placed onto the bag and a multi-point thermocouple will be inserted through the pad into the bag. We will simulate the moist heat of a bread proofing oven or dry heat environment of a convection oven in the laboratory 132. Thermocouples also will be placed inside ovens to accurately measure the "come-up" times and ensure accurate time and temperature measurements can be made. Standard thermal death time measurements will be made using these inoculated coupons in both moist and dry heating mediums. Coupons will be removed after

6 different combinations of time/temperature. The biocidal effectiveness of the heat process to destroy the biofilms will be assessed as above. Two sets of plates will be prepared and duplicate dilutions will be plated on MOX agar (for enumerating non injured cells) and on TSAYE to allow any injured but not dead L. monocytogenes to resuscitate and grow.
**e.** Effectiveness of cloths on removal of contaminants. Surfaces of Formica, plastic, stainless steel or other will be marked off in 5.5 X 5.5 cm grids. Surface will be cleaned with Alconox detergent, rinsed three times with deionized water and then rinsed with freshly prepared 10% bleach, and then rinsed with sterile distilled water. Surface is then placed in Biosafety Cabinet. Lm cocktail (0.5 mL) is pipetted onto surface, spread evenly with L-spreader, and allowed to dry for 2 h. Test cloths (cut in 5 X 5 cm sq) will be dampened and placed in autoclave sleeves and autoclaved. Sterile test cloths will be wiped across inoculated areas 5 times vertical and 5 times horizontal in attempt to remove contaminant. Latex gloves used for holding cloths are changed after each cloth. Cloths are disposed of in autoclave bags. To test for contaminant removal, sterile calcium alginate swabs are wiped 10 times vertically, 10 times horizontally on the gridded surface and placed into tubes containing 9 mL sterile peptone water. Samples are serially diluted and plated onto MOX agar. Plates are incubated for 48 h at 37 °C, and enumerated.

**The information requested above can be entered directly or cut & pasted into the space provided, or can be provided as an attached word document. If you provide an attachment, please indicate "See Attached" and list the file name(s) in the space below:**

Click here to enter text.

**PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:**
**List all personnel (including PI and Co-PI) to be involved in this project:**

| **Name (First and Last) - Position (Title, academic degrees, certifications, and field of expertise)** | **Qualifications/Training/Relevant Experience (Describe previous work or training with biohazardous and/or recombinant DNA; include Biosafety Levels )** |
|---|---|
| Example: Bob Biohazard - Associate Professor, PhD-Microbiology | 14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice. |
| Philip G. Crandall (Co-PI), Ph.D., Professor, Food Science | 10 years working with BSL 2 pathogens |
| Steven C. Ricke (Co-PI), Ph.D., Donald "Buddy" Wray Chair in Food Safety and Director, Center for Food Safety in the Institute of Food Science and Engineering | 20 years working with anaerobic bacteria and food-borne pathogens |
| Elizabeth M. Martin, PhD plant pathology, Program Tech, Bio/Ag Engineering | Worked over 20 years with plant viruses. Worked 2 years with poultry viruses, bacteria and mycoplasma (BL2-3). Worked 10 years with food borne pathogens (BL2) |
| Corliss O'Bryan, Post Doctoral Associate Ph.D. | 30 years working with BL1 and Bl2 bacteria including Salmonella, E. coli O157:H7, Listeria monocytogenes. |
| Ok Kyung Koo, Ph.D., Post Doctoral Associate, Food Science | 6 years working with BL2 bacteria including Salmonella, E. coli O157:H7, Listeria monocytogenes and BL2 mammalian |

| | |
|---|---|
| | cell cultures |
| Nathan Jarvis, Ph.D. candidate | To be trained on working with Listeria monocytogenes |
| Mallory Eggleton, Undergrad | One year working with Listeria monocytogenes |

**Additional Personnel Information (if needed):**

Click here to enter text.

**List all the laboratories/facilities where research is to be conducted:**

| Building: | Room #: | Category: | *Signage Correct? |
|---|---|---|---|
| BAEG 300 | 207 | **Laboratory** | **Yes** |
| BAEG 300 | 100 | **Autoclave/BioStorage** | **Yes** |
| BAEG 300 | 117 | **Cold Room** | **Yes** |
| BAEG 300 | 208 | **Cold Room** | **Yes** |
| Biomass Res. Center | 132 | **Laboratory** | **Yes** |
| Biomass Res. Center | 102 | **Laboratory** | **Yes** |
| Biomass Res. Center | 101 | **Autoclave/BioStorage** | **Yes** |
| | | Choose an item. | Choose an item. |
| | | Choose an item. | Choose an item. |
| | | Choose an item. | Choose an item. |

*** Biohazard signs are required for entrances to Biosafety Level 2 (including Animal Biosafety Level 2) areas. EH&S will supply these signs. If an updated biohazard sign is required, please indicate the location and what agents/organisms/hazards should be listed on the sign:**

Click here to enter text.

**Additional Facility Information (if needed):**

Click here to enter text.

**SAFETY PROCEDURES:**
Please indicate which of the following personal protective equipment (PPE) will be used to minimize the exposure of laboratory personnel during all procedures that require handling or manipulation of registered biological materials.

**Gloves:**

☑ Latex
☐ Nitrile
☐ Other
☐ Vinyl
☐ Leather
Specify: Click here to enter text.

**Face & Eye Protection:**

- ☐ Face Shield
- ☐ Safety Goggles
- ☑ Safety Glasses
- ☐ Other — Specify: Click here to enter text.

**Clothing Protection:**

- ☑ Re-usable Lab Coat
- ☐ Re-usable Coverall
- ☐ Disposable Clothing Protection
- ☐ Other — Specify: Click here to enter text.

**Dirty or contaminated protective clothing cleaning procedures:** (Check all that apply)

- ☑ Autoclaved prior to laundering or disposal
- ☐ Laundered on site using bleach
- ☐ Laundered by qualified commercial service
- ☐ Other — Specify: Click here to enter text.

**Outline procedures for routine decontamination of work surfaces, instruments, equipment, glassware and liquid containing infectious materials. Autoclaving or using fresh 10% bleach as a chemical disinfectant are preferred treatments; please specify and justify any exceptions:**

Work surfaces will be decontaminated with a freshly prepared 10% bleach solution before and after working. Exception is biosafety cabinets which will be disinfected before and after use with Lysol® No Rinse Sanitizer in order to avoid the corrosiveness of the bleach on the metal of the biosafety cabinets. Instruments and equipment will be decontaminated by wiping down with 10% bleach. Paper towels used for these purposes will be discarded in biohazard bags. Glassware, waste, and disposable tubes will be autoclaved under standard conditions (15 psi, 121 C, 20 min). Disposable items (pipette tips, pipets, etc) will be discarded into 10% bleach. After 30 minutes it will be permissible to place these items in a biohazard bag for autoclaving before disposal.

**Describe waste disposal methods to be employed for all biological and recombinant materials. Include methods for the following types of waste:** (ref: UofA BiosafetyManual )

**Sharps:**

Placed into 10% bleach solution for decontamination followed by discarding into sharps waste container

**Cultures, Stocks and Disposable Labware:**
Placed into biohazard bags and autoclaved before disposal. Liquids will be disposed of in drains after autoclaving. Disposable glass will be placed in glass disposal after autoclaving.

**Pathological Waste:**

Liquid biological waste will always be discarded into freshly made 10% bleach and then autoclaved for decontamination treatment before it is discarded. Other biological waste will be placed carefully into biohazard waste bags, autoclaved at 15 psi, 1210C for 20 min.

**Other:**

Click here to enter text.

**Autoclave(s), to be used in this project, location(s) and validation procedures:**

Biomass Res. Ctr. Room 101 and BAEG room 100: Autoclaves are checked monthly using SteriGage test strips (3M) and SporAmpule vials to ensure autoclaves completely sterilize all bacterial life forms including spores.

**Will biological safety cabinet(s) be used?**
**Yes**

**If yes, please provide the following information:**

| **Make/Model** | **Serial Number** | **Certification Expiration** | **Location** (bldg/room) |
|---|---|---|---|
| Biosafety Cabinet Level II ThermoForma Model 1186 | 100663 | **11/30/2011** | Biomass Res. Center, Room 132 |
| Biosafety Cabinet Level II FormaScientific Model 1000 | 13324-539 | **11/30/2011** | Biomass Res. Center, Room 132 |
| Biosafety Cabinet Level II FormaScientific Model 1126 | 12118-128 | **11/30/2011** | Biomass Res. Center, Room 132 |
| Labconco – Class II | 040520458 AB | **11/30/2011** | Bldg 300, Bio/Ag Eng. Research Lab, Room 207 |
| Click here to enter text. | Click here to enter text. | Click here to enter a date. | Click here to enter text. |

**Additional Biological Safety Cabinet Information (if needed):**
Click here to enter text.

**Indicate if any of the following aerosol-producing procedures will occur:** (check all that apply)

- ☑ Centrifuging
- ☐ Blending
- ☑ Sonic Disruption
- ☐ Dissection
- ☐ Stomacher
- ☐ Other
- ☐ Grinding
- ☑ Vigorous Shaking or Mixing
- ☑ Pipetting
- ☐ Innoculating Animals Intranasally

Describe: Click here to enter text.

**Describe the procedures/equipment that will be used to prevent personnel exposure during aerosol-producing procedures:**

All pipetting of infectious material will take place in the biological safety cabinet. Mechanical pipetting devices will be used. Lab coats buttoned over street clothes, gloves and goggles will be worn. All needed materials will be placed in the biological safety cabinet before work begins. Sash of the cabinet will be lowered and all movements will be slow to avoid disruption of the air currents. Centrifuged cultures will be contained in a closed Eppendorf tube or contained in screw-capped polypropylene or polystyrene tubes with gasket seals to prevent aerosol exposure. Cultures to be vortexed will be contained in screw-capped polypropylene or polystyrene tubes, and vortexing will be done within the biological safety cabinets. Sonicating will be done within the biosafety cabinet or within an enclosure on the bench top.

**EMERGENCY PROCEDURES:**

**In the event of personnel exposure (e.g. mucous membrane exposure or parenteral inoculation), describe what steps will be taken including treatment, notification of proper supervisory and administrative officials, and medical follow up evaluation or treatment:**

In the event of accidental exposure of personnel the person exposed should notify the laboratory supervisor immediately. Treatable exposures will be treated by use of the first aid kit containing antimicrobial agents. Mucous membrane exposure or puncture with contaminated material will result in the person being taken to the Health Center for prophylactic antibiotic therapy.

**In the event of environmental contamination, describe what steps will be taken including a spill response plan incorporating necessary personal protective equipment (PPE) and decontamination procedures.**

For a spill inside the biological safety cabinet, alert nearby people and inform laboratory supervisor. Safety goggles, lab coat buttoned over street clothes and latex gloves should be worn during clean up. If there are any sharps they will be picked up with tongs, and the spill covered with paper towels. Carefully pour disinfectant (freshly made 10% bleach) around the edges of the spill, then into the spill without splashing. Let sit for 20 minutes. Use more paper towels to

wipe up the spill working inward from the edge. Clean the area with fresh paper towels soaked in disinfectant. Place all contaminated towels in a biohazard bag for autoclaving. Remove personal protective clothing and wash hands thoroughly.

For a spill in the centrifuge turn off motor, allow the machine to be at rest for 30 minutes before opening. If breakage is discovered after the machine has stopped, re close the lid immediately and allow the unit to be at rest for 30 minutes. Unplug centrifuge before initiating clean up. Wear strong, thick rubber gloves and other personal protective equipment (PPE) before proceeding with clean up. Flood centrifuge bowl with disinfectant. Place paper towels soaked in a disinfectant over the entire spill area. Allow 20 minute contact time. Use forceps to remove broken tubes and fragments. Place them in a sharps container for autoclaving and disposal as infectious waste. Remove buckets, trunnions and rotor and place in disinfectant for 24 hours or autoclave. Unbroken, capped tubes may be placed in disinfectant and recovered after 20 minute contact time or autoclaved. Use mechanical means to remove remaining disinfectant soaked materials from centrifuge bowl and discard as infectious waste. Place paper towels soaked in a disinfectant in the centrifuge bowl and allow it to soak overnight, wipe down again with disinfectant, wash with water and dry. Discard disinfectant soaked materials as infectious waste. Remove protective clothing used during cleanup and place in a biohazard bag for autoclaving. Wash hands whenever gloves are removed.

For a spill outside the biological safety cabinet or centrifuge have all laboratory personnel evacuate. Close the doors and use clean up procedures as above.

**TRANSPORTATION/SHIPMENT OF BIOLOGICAL MATERIALS:**

**Transportation of Biological Materials:** The Department of Transportation regulates some biological materials as hazardous materials; see 49 CFR Parts 171 - 173. Transporting any of these regulated materials requires special training for all personnel who will be involved in the shipping process (packaging, labeling, loading, transporting or preparing/signing shipping documents).

**Will you be involved in transporting or shipping human or animal pathogens off campus?**
**No**

**If yes, complete the remaining:**

- ☐ Cultures of Human or Animal Pathogens
- ☐ Environmenatl samples known or suspected to contain a human or anumal pathogen
- ☐ Human or animal material (including excreta, secreta, blood and its components, tissue, tissue fluids, or cell lines) containing or suspected of containing a human or animal pathogen.

**Transportation/Shipment Training:** Have any project personnel who will be involved in packaging, labeling, completing, or signing shipping documents received formal training to ship infectious substances or diagnostic specimens within the past 3 years?
Choose an item.

**If yes, please provide the following information:**

| Name | Date Trained | Certified Shipping Trainer |
|---|---|---|
| Click here to enter text. | Click here to enter a date. | Click here to enter text. |
| Click here to enter text. | Click here to enter a date. | Click here to enter text. |
| Click here to enter text. | Click here to enter a date. | Click here to enter text. |
| Click here to enter text. | Click here to enter a date. | Click here to enter text. |
| Click here to enter text. | Click here to enter a date. | Click here to enter text. |

****Fill out a copy of this form for each agent used****

**IBC Number:** 08028 renewal

**Principal Investigator:** Dr. Philip G. Crandall

**Agent:** ***(Genus & Species)*** Listeria monocytogenes

**Agent Type:**

- ○ Viral
- ○ Chlamydial
- ◉ Bacterial
- ○ Fungal
- ○ Parasitic
- ○ Prion
- ○ Rickettsial
- ○ Other Describe: Click here to enter text.

**Agent Strain:** ***(Check all that apply)***

- ☐ Human Pathogen (not animal)
- ☐ Animal Pathogen (not human)
- ☑ Human / Animal Pathogen
- ☐ Plant Pathogen
- ☑ Opportunistic Pathogen

**Host Range:** Mammals, birds, fish, crustaceans and insects

| | |
|---|---|
| Disease or Toxin Produced: | Opportunistic pathogen manifested in the elderly, in neonates and or among immunocompromised individuals as meningoencephalitis and/or septicemia; inapparent infection at all ages with consequence only during pregnancy; perinatal infections occur transplacentally and can result in abortion, stillbirth; meningitis, endocarditis, septicemia, and disseminated granulomatous lesions in adults |
| Route of Transmission: | In neonates, transmission from mother to fetus in utero or during passage through infected birth canal; direct contact with infectious material or soil contaminated with infected animal feces can result in papular lesions on hands and arms; ingestion of contaminated food; inhalation of the organism is possible. |
| Virulence (lowest infective dose) or toxicity ($LD_{50}$): *(specify animal model e.g. $LD_{50}$ Rat)* | Not known |

**Are there any vaccinations, skin tests or other medical prophylactic treatments or medical surveillance necessitated by work with this agent?**

No

**If yes, please explain:**

Click here to enter text.

**Will infectious aerosols be generated?**

Yes

**Work with this agent will be conducted:** *(Check all that apply)*

- ☐ On the Lab Bench
- ☐ In a Clean Bench
- ☐ In a Clean Room
- ☐ Other Specify: Click here to enter text.
- ☐ In a Fume Hood
- ☐ In a Glove Box
- ☑ In a Biological Safety Cabinet

University of Arkansas
**Institutional Biosafety Committee** **IBC number**
**Registration for Research Projects** *For Committee Use Only*

## Form 9: NOTICE TO PAT WALKER HEALTH CENTER

**Principal Investigator Name:** First M.I. Last

**Principal Investigator Contact Information:** Office Location:

Office Phone: Cell Phone:

E-Mail:

### List of BSL-2 or above agents being used and their toxins:

**1.**

**Agent** (*Genus & Species*)**:**

**Agent Type:**

Viral Bacterial Parasitic Rickettsial Chlamydial Fungal Prion

Other Describe:

**Agent Strain:** *(check all that apply)*

Human Pathogen (not animal) Human/Animal Pathogen Opportunistic Pathogen

Animal Pathogen (not human) Plant Pathogen

**Disease or Toxin Produced:**

**2.**

**Agent** (*Genus & Species*)**:**

**Agent Type:**

Viral Bacterial Parasitic Rickettsial Chlamydial Fungal Prion

Other Describe:

**Agent Strain:** *(check all that apply)*

Human Pathogen (not animal) Human/Animal Pathogen Opportunistic Pathogen

Animal Pathogen (not human) Plant Pathogen

**Disease or Toxin Produced:**

**3.**

**Agent** (*Genus & Species*)**:**

**Agent Type:**

Viral Bacterial Parasitic Rickettsial Chlamydial Fungal Prion

Other Describe:

**Agent Strain:** *(check all that apply)*

Human Pathogen (not animal) Human/Animal Pathogen Opportunistic Pathogen

Animal Pathogen (not human) Plant Pathogen

**Disease or Toxin Produced:**

UNIVERSITY OF
ARKANSAS

*Office of Research Compliance*

May 12, 2011

MEMORANDUM

TO: Dr. Philip Crandall

FROM: W. Roy Penney
Institutional BioSafety Committee

RE: IBC Protocol Approval

IBC Protocol #: 08028

Protocol Title: "Cost Effective Treatments to Minimize Listeria monocytogenes Cross Contamination of Ready-To-Eat Meats by the In-Store Deli Meat Slicer"

Approved Project Period: Start Date: June 30, 2008
Expiration Date: June 29, 2014

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 08028, "Cost Effective Treatments to Minimize Listeria monocytogenes Cross Contamination of Ready-To-Eat Meats by the In-Store Deli Meat Slicer". You may continue your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

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